Identification and characterization of retinoic acid-induced morphological and electrophysiological changes in an invertebrate nervous system

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Abstract

The vitamin A metabolite, retinoic acid (RA) is known to play an important role in the development, patterning and regeneration of nervous tissue, both in the embryo and in the adult. Classically, RA is known to mediate the transcription of target genes through the binding and activation of its nuclear receptors: the retinoic acid receptors (RARs) and retinoid X receptors (RXRs). Recently, mounting evidence from many animal models has implicated a number of RA-mediated effects operating independently of gene transcription, and thus highlights novel, nongenomic actions of RA. For example, recent work utilizing cultured neurons from the pond snail, *Lymnaea stagnalis,* has shown that RA can elicit a regenerative response, growth cone turning, independently of "classical" transcriptional activation. While this work illustrates a novel regeneration-inducing effect in culture, it is currently unknown whether RA also induces regeneration *in situ*. This study has sought to determine RA's regenerative effects at the morphological and molecular levels by utilizing an *in situ* approach focusing on a single identified dopaminergic neuron which possesses a known "mapped" morphology within the CNS. These studies show, for the first time in an invertebrate, that RA can increase neurite outgrowth of dopaminergic cells that have undergone a nerve-crush injury. Utilizing Western blot analysis, it was shown that this effect appears to be independent of any changes in whole CNS expression levels of either the RAR or RXR. Additionally, utilizing immunohistochemistry, to examine protein localization, there does not appear to be any obvious changes in the RXR expression level at the crush site. Changes in cell morphology such as neurite extension are known to be modulated by changes in neuronal firing activity. It has been previously shown that exposure to RA over many days can lead to changes in the electrophysiological properties of cultured *Lymnaea* neurons; however, no studies have investigated whether short-term exposure to RA can elicit electrophysiological changes and/or changes in firing pattern of neurons in *Lymnaea* or any other species. The studies performed here show, for the first time in any species,

that short-term treatment with RA can elicit significant changes in the firing properties of both identified dopaminergic neurons and peptidergic neurons. This effect appears to be independent of protein synthesis, activation of protein kinase A or phospholipase C, and calcium influx but is both dose-dependent and isomer-dependent. These studies provide evidence that the RXR, but not RAR, may be involved, and that intracellular calcium concentrations decrease upon RA-exposure with a time course, dose-dependency and isomer-dependency that coincide with the RA-induced electrophysiological changes. Taken together, these studies provide important evidence highlighting RA as a multifunctional molecule, inducing morphological, molecular and electrophysiological changes within the CNS, and highlight the many pathways through which RA may operate to elicit its effects.

List of Acronyms

Acronym	Definition
$[Ca]_i$	Intracellular calcium level
9cis RA	9-*cis* retinoic acid
AM	acetoxymethyl
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
atRA	*all-trans* retinoic acid
BAPTA	1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
CaMKKα	calcium/calmodulin protein kinase α
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
CM	Conditioned media
CNS	Central nervous system
CYP26	Cytochrome P450 protein 26
DM	Defined medium
DMSO	Dimethyl sulfoxide
EC_{50}	Half maximal excitatory concentration
EDTA	Ethylenediaminetetraacetic acid
ERK	Extracellular-signal-regulated kinase
EtOH	Ethanol
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HL-60	Human promyelocytic leukemia cells
IC_{50}	Half maximal inhibitory concentration
IP3	Inositol trisphosphate
K_{Ca}	Calcium dependent potassium
MEK	Mitogen activated extracellular-signal-regulated kinase
mRNA	Messenger ribonucleic acid
PBS	Phosphate buffered saline
PKA	Protein kinase A
PKAi	Protein kinase A inhibitor Rp-Adenosine 3'5'cyclic monophosphoraite
PKC	Phospho kinase C
PLC	Phospholipase C
PLCi	Phospholipase C U-73122
PMSF	Phenylmethanesulfonylfluoride
RA	Retinoic acid
RALDH	Retinaldehyde dehydrogenase
RAR	Retinoic acid receptor
RARE	Retinoic acid response element
RMP	Resting membrane potential
RPeD1	Right pedal dorsal 1
RXR	Retinoid X receptor

S.E.M.	Standard error of the mean
SDS	Sodium dodecyl sulfate
TTNPB	(E)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthylenyl)-1 -propenyl] benzoic acid
VF	Visceral F

Chapter 1
Introduction & Literature Review

1.01 – General Introduction

The biologically active metabolite of vitamin A, retinoic acid (RA) has received much attention within the scientific community for its role in a multitude of cellular and developmental processes. Currently, RA has been implicated in such processes as developmental patterning of the central nervous system (CNS) and limb tissue, neuronal differentiation, cell proliferation, and in the adult, learning and memory, immune function and neuronal regeneration. Perturbations to the RA signaling pathway are thought to play a role in the progression of numerous ailments and diseases such as cancer (breast and prostate), age related learning and memory deficits and many neurological diseases that affect dopaminergic neurons such as Alzheimer's disease, Parkinson's disease, Huntington's disease, depression and schizophrenia. Additionally, RA has been thought to play a critical role in the regeneration of neural tissue, either in response to injury or during disease-induced degeneration. In general, studies into RA's roles in these diseases as well as in response to injury have focused largely on RA's ability to influence gene expression, and thus research has focused on RA in the context of a transcriptional activator. However, recent work from many disciplines has begun to illustrate RA's capacity to operate through a number of novel, non-transcriptional mechanisms. Evidence is emerging implicating a number of nongenomic (ie: nontranscriptional) roles for the retinoid receptors as well as the ability for RA to bind directly to intracellular messengers. This highlights the multiple roles RA can have, and its ability to operate in tandem through multiple mechanisms within cells both transcriptionally and non-transcriptionally.

The emergence of a number of novel roles RA can play outside that of transcriptional activation underscores the need to examine new, non-transcriptional effects RA may have in various disease and/or injury states. In particular, it is currently unknown whether the non-transcriptional

effects of RA play a role a role in the progression of neurodegnererative diseases and response to neural injury. Moreover, since aberrant RA activity is thought to play a role in a number of neurological diseases, particularly those affecting dopaminergic neurons (Krezel et al., 1998; Bremner and McCaffery, 2008; Katsuki et al., 2009; Ulusoy et al., 2011), a clearer understanding of the roles RA plays, both transcriptionally and non-transcriptionally may provide valuable insight into the use of RA as a therapeutic strategy in the treatment of these diseases.

Growing evidence from the literature has shown that the firing pattern of neurons may modulate morphological responses and thus modulate the regeneration of damaged neurons. Thus, the electrophysiological properties of neurons would appear to be an important component in neural regeneration. Despite being well studied for its morphological role during regeneration, very little is known about the mechanisms required for RA to elicit its regenerative effects. In particular, it is unknown whether RA elicits its regenerative effects in part through changes in the firing properties of injured neurons. Surprisingly, no studies exist directly investigating whether short-term exposure to RA may alter the firing properties of neurons.

The main aim of my thesis is to first utilize a molluscan model with a single identified dopaminergic neuron to study the effects of RA on regeneration. Given that RA is thought to play a role in a number of neurological diseases affecting dopaminergic neurons, these studies may provide valuable insight into the role RA may have in these disease states. My second main aim is to investigate the effects of RA on the electrophysiological properties of neurons with the purpose of determining whether such changes may ultimately play a role in RA's effects on regeneration in the nervous system. In the following sections I provide an overview of the roles

RA has in development and regeneration, and will then provide evidence highlighting new, nontranscriptional actions of RA within the cell.

1.02 – Retinoic Acid Metabolism

Typically, vitamin A (retinol) is obtained in the form of retinal esters from the dietary intake of animal tissues or is ingested in the form of plant carotenoids, which are later converted into retinol in the gut or liver (Theodosiou et al., 2010). Retinol is then transported to the cytoplasm of individual cells, where it is metabolized to retinal and then to RA via the enzymes alcohol dehydrogenase and retinaldehyde dehydrogenase (RALDH), respectively (Theodosiou et al., 2010). There are two predominate endogenous isomers of RA, *all-trans* RA (atRA) and *9-cis* RA (9*cis* RA), with atRA typically being more abundant in most animal species studied to date (Theodosiou et al., 2010). It has been widely accepted that the retinoic acid receptors (RARs) and retinoid X receptors (RXRs) bind to atRA and 9*cis* RA, respectively (Figure 1.1). Additionally, while it is also known that the RAR can bind with 9*cis* RA, some work has suggested that the RXRs bind 9*cis* RA with higher affinity than that of atRA (Theodosiou et al., 2010). To date there are three known classes of RARs and RXRs, denoted α, β and γ (McGrane, 2007; Theodosiou et al., 2010). Additional subtypes of α, β and γ class receptors have also been found in various animals, denoted as α1, α2, β1, β2, etc. (McGrane, 2007; Theodosiou et al., 2010). Once bound to RA, these receptors can then heterodimerize or homodimerize and bind to DNA at enhancer sites known as retinoic acid response elements (RAREs) to elicit genomic effects; more specifically, the activation of gene expression at the transcriptional level (McGrane, 2007; Theodosiou et al., 2010). Such RA-regulated genes can include, for example, the *Hox* (Theodosiou et al., 2010) and Wnt (Elizalde et al., 2011) family of genes, which are known to play a critical role in the axial patterning of tissue within the developing embryo. The differential expression of retinoid receptor subtypes, for example the RARβ, is thought to play a

critical role in a number of processes such as newt spinal cord regeneration (Dmetrichuk et al., 2005), limb development (Williams et al., 2009) and in its absence, the induction of breast cancer (Sun et al., 2011). Additionally, both the RARs and RXRs have been shown to bind with a number of other nuclear receptors including hormone receptors and vitamin D receptors, influencing the transcription of a number of other gene families both during development and in the adult (Brtko and Dvorak, 2011). Lastly, the breakdown and clearance of RA occurs through the CYP26 family of proteins. These enzymes break down both atRA and 9*cis* RA isomers into oxidized products which can then be removed as waste (Thatcher and Isoherranen, 2009).

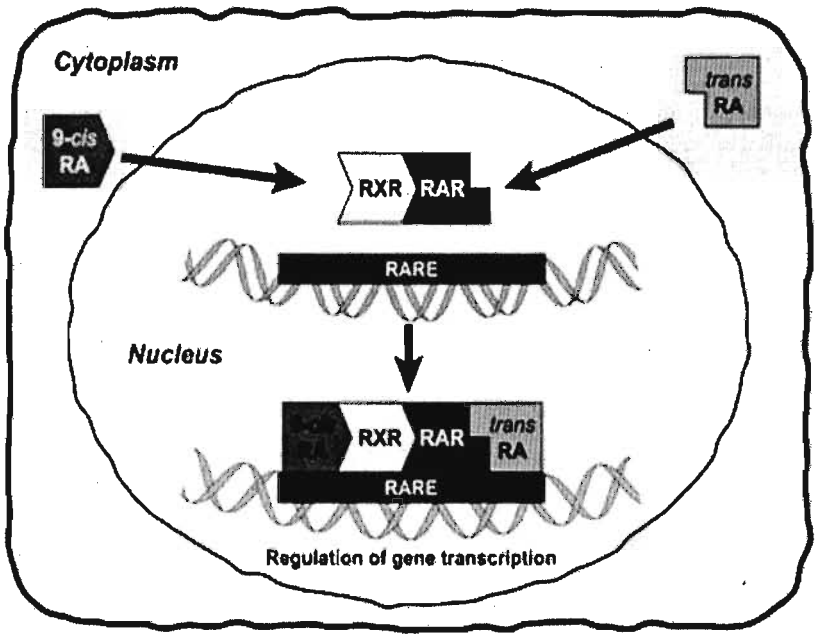


Figure 1.1. Schematic of RA-induced transcriptional activation of target genes. Both the RAR and RXR receptors, once bound to RA, can either homo or heterodimerize and bind to target genes containing a RARE sequence. Once bound to a RARE, the activated retinoid receptors aid in the transcriptional activation of the target gene. Adapted from Linus Pauling Institute, Oregon State University.

1.03 - RA in Development and Limb Regeneration

RA has been extensively studied for its role in limb formation (Lee et al., 2004; Reijntjes et al., 2010) and neuronal patterning during vertebrate development (for reviews see Dolle (2009), Maden (2007) and Zile, (2001)). During CNS and limb development, a RA concentration gradient is established in the developing tissue. This gradient serves as a positional guide within the CNS or limb and aids in the proper patterning of the target tissue. While classically examined in the context of development, RA has also received similar focus for its role in the regeneration of adult nervous tissue (for reviews see Mey and McCaffery (2004); Mey (2006)), owing partly to the ideology that regeneration recapitulates developmental processes. Numerous studies have shown that modification of the RA concentration gradient either within the developing animal or the regenerating limb, typically achieved via application of exogenous RA, often leads to developmental abnormalities. For example, exposing developing zebrafish embryos to exogenously applied RA has been shown to induce bifurcation (doubling) of the retina (Hyatt et al., 1992). Similar work utilizing the regenerating tail and hind limb of tadpoles has shown that excessive, as well as low RA concentrations can induce multiple duplications of the hind limb, a condition known as "super-regeneration" (Maden and Hind, 2003). These limbs have been shown to sprout from the developing end of both the hind limb and tail. Thus, perturbation of the RA signaling pathway during embryonic development and/or limb regeneration leads to the disruption of patterning molecules within the organism and ultimately to wide scale disruption of the structural organization of tissues within the embryo.

While excessive RA levels in the developing embryo and/or regenerating limb have been shown to result in large scale perturbations, a number of studies have also shown that the reduction of endogenous RA levels often leads to inhibition of limb formation. For example, studies

performed in zebrafish (Vandersea et al., 1998) as well as chick (Tanaka et al., 1996) have shown that application of citral, a synthetic inhibitor of RA synthesis, leads to the absence of pectoral fins and wing buds, respectively. In both cases, application of RA was able to rescue embryos from the citral-induced limb deformities. While these studies only represent a fraction of the work performed within the field of development and regeneration, there is an overwhelming amount of support showing a role for RA in CNS and limb patterning. Importantly, it would appear that there is a critical level of RA which is required for the proper development of the target tissue, since excessive as well as insufficient RA levels lead to large scale deformities. The examples given above illustrate the "classical" roles of RA as a transcriptional activator, whereby it is thought that in these situations RA is eliciting its effects exclusively through the activation of retinoid receptors which act to regulate gene expression.

1.04 - RA's Involvement in Neural Regeneration

Historically, most work has investigated the role of RA in the context of development and limb regeneration, a rapidly emerging field of work has focused specifically on RA's regenerative capacity during neuronal damage. With the general view that regeneration recapitulates developmental processes, many studies have focused on the ability of RA to aid and support the regeneration of injured nervous tissue (for review see Mey (2006)). As with previous developmental studies, the bulk of this work has focused on the "classical retinoid signaling pathway" and, thus, on RA's role as a transcriptional activator. The next section will summarize two main findings implicating RA as a regeneration-inducing molecule, particularly within neural tissue. The first is that the retinoid signaling pathway is upregulated (and thus activated) in response to neuronal injury; the second is that application of exogenous RA can enhance the regeneration of damaged neural tissue.

RA is largely thought to act in a regenerative capacity in response to neuronal damage. Numerous studies have shown that the classical retinoid signaling pathway is activated in response to either peripheral or central nerve injury. For example, the retinoid receptors have been shown to be dramatically upregulated in rats exposed to a sciatic nerve injury (Zhelyaznik and Mey, 2006) or spinal cord injury (Agudo et al., 2010; Schrage et al., 2006). Similar results have also been found in zebrafish exposed to a spinal cord lesion (Reimer et al., 2009). In addition to the retinoid receptors, RALDH has been found to be rapidly upregulated in goldfish exposed to optic nerve damage (Nagashima et al., 2009). This shows that in addition to increased retinoid receptor expression, the RA synthesis machinery is also dramatically increased in response to neuronal injury. In summary, these studies illustrate a consistent trend which suggests that the classical RA signaling pathway (both the retinoid receptors and the RA synthesis machinery) is activated and upregulated in response to neural damage.

Having implicated the classical retinoid signaling pathway's involvement in a regenerative response to neural damage, other studies have sought to test directly whether exogenous application of RA or synthetic analogues can induce or enhance neural regeneration. Such work has shown that activation of the retinoid signaling pathway can protect neurons from degeneration in rat (Katsuki et al., 2009) as well as promote neurite outgrowth in damaged mouse (So et al., 2006) and newt (Dmetrichuk et al., 2005) neurons. Importantly, it has been shown in rats that application of a synthetic retinoid can lead to functional recovery *in vivo* (Agudo et al., 2010). Taken together, these findings suggest that application of RA may increase the regenerative abilities of damaged neurons and promote neuronal recovery.

In summary, the classical RA signaling pathway is dramatically upregulated in response to neuronal damage. Application of RA or synthetic RA analogues can promote, induce and enhance neurite outgrowth and have the capacity to lead to functional recovery. Importantly, this work has typically examined the response to RA many days after injury, implicating RA's role in regeneration rather than injury-response; commonly described as an initial degeneration of injured neurons which typically occurs within the first 48 hours after injury (Gaudet et al., 2011). Interestingly, despite growing evidence of RA's ability to operate though a number of "nonclassical" (ie: nontrasncriptional) pathways, very little work exists examining whether these pathways are utilized in response to neural injury.

1.05 - Evidence for Novel RA Interactions

A growing body of literature has recently shed light on RA's capacity to operate outside that of the classical retinoid signaling pathway. In other words, a number of effects elicited by RA appear to operate through means other than that of transcriptional activation of genes containing a RARE sequence. Such work can be compiled into three main categories. First, some studies have documented a number of nongenomic (non-transcriptional) actions of the retinoid receptors whereby RA-induced effects are mediated through retinoid receptors, but appear to be independent of transcriptional activation(Han et al., 2009; Maghsoodi et al., 2008; Zeng et al., 2006; Zhelyaznik and Mey, 2006). Secondly, some literature has documented situations where RA exposure can elicit dramatic changes in the activity of intracellular signaling proteins such as protein kinase A (PKA) (Rochette-Egly et al., 1995; Santos and Kim, 2010; Saito et al., 2010) and phospholipase C (PLC) (Geny et al., 1991; Jetten and Shirley, 1985; Haase et al., 1997), highlighting RA's ability to target specific intracellular messengers within cells. Lastly, a number of studies have shown that RA may have the capacity to alter the electrical properties of

cells (Romero-Sandoval et al., 2006; Arcangeli et al., 1998; Zhang and McMahon, 2000) as well as modulate changes in the intracellular calcium concentration (Gao et al., 1998; Short et al., 1991; Shyu et al., 2003), potentially leading to numerous physiological effects within neurons. The following sections briefly discuss recent work illustrating examples of RA operating outside that of the classical retinoid signaling pathway.

I. Nongenomic Actions of the Retinoid Receptors

Two main lines of evidence are currently emerging suggesting that the retinoid receptors may also play important roles outside that of transcriptional activation. First, evidence is emerging which documents the presence of retinoid receptors in distal neuronal regions, such as growth cones, axons and dendrites. For example, in response to neuronal damage both the RAR and RXR have been found to co-localize in regenerating rat axons, rather than in the cell nuclei (as would be expected of transcriptional activators) (Zhelyaznik and Mey, 2006). Similar work has shown the dendritic localization of the RAR in rat hippocampal neurons (Maghsoodi et al., 2008) and in human cell lines, the cytoplasmic localization of both the RAR (Han et al., 2009) and RXR (Zeng et al., 2006). Furthermore, the application of plant extracts (Zeng et al., 2006) or media containing differing amounts of serum (Han et al., 2009) can result in the translocation of the retinoid receptors out of the nucleus into the cytoplasm, which (as suggested by the authors) could suggest that the retinoid receptors may play important, nongenomic roles outside of the nucleus. Lastly, experiments utilizing the pond snail *Lymnaea stagnalis* have shown evidence for the cytoplasmic localization of the RXR in extending neurites and growth cones of cultured neurons (Carter et al., 2010). Taken together, these studies provide substantial evidence that retinoid receptors are often found outside the nucleus, which would be unexpected if their role was only that of a transcriptional activator. Their presence within the cytoplasm, and particularly

in the distal regions of neurons could suggest the possibility that these receptors may also play other, nongenomic roles within local microdomains of cells.

In addition to studies showing non-nuclear localization of retinoid receptors, new evidence has emerged suggesting that the retinoid receptors can play important nongenomic roles regulating the response to RA. For example, work in rat has shown that the RAR can act locally at dendrites to regulate the translational expression of target mRNAs (Maghsoodi et al., 2008; Poon and Chen, 2008; Sidell et al., 2010). Cumulatively, this work has shown that the RAR translationally represses the expression of target mRNAs. This translational repression can be released upon the binding of RA to the RAR. Supporting work has shown that the RAR can bind to numerous translation factors, including RNA transport proteins and RNA translation factors, which are used in dendritic protein synthesis (Chen et al., 2008), strengthening the notion of the RAR acting nongenomically. Lastly, as will be discussed in more detail in a later section, studies utilizing cultured neurons from *Lymnaea* have demonstrated that application of RA (Dmetrichuk et al., 2006a) as well as a RXR pan-agonist (Carter et al., 2010) can direct neurite outgrowth in neurites lacking a cell body (and thus a nucleus), adding further support to the notion that the retinoid receptors can play nongenomic roles. Taken together this work shows that some RA-mediated effects can be modulated via the nongenomic actions of retinoid receptors.

II. Modulation of Signaling Pathways

Substantial work has shown that RA also has the ability to directly modulate a number of intracellular signaling pathways and suggests that RA does so without any involvement of the retinoid receptors. Typically, these studies have shown that RA exposure results in the increased

activity of specific proteins within a signaling cascade, such as PKA (Saito et al., 2010, Rochette-Egly et al., 1995; Santos and Kim, 2010) or PLC (Geny et al., 1991; Jetten and Shirley, 1985; Haase et al., 1997). Thus this work highlights the ability for RA to modulate a number of signaling cascades within a cell which are known to play important roles in regeneration, thus extending its capacity to elicit change well beyond that of a transcriptional activator.

Substantial work has shown that RA may modulate the activity of PKA. For example, RAR-mediated transcriptional activity has been shown to be increased in a PKA-dependent manner following application of RA (Saito et al., 2010). This RA-induced increase was blocked by application of a PKA antagonist, suggesting that RA may regulate PKA activity which in turn can modulate the activity of the RAR (Saito et al., 2010). Supporting work in cell culture has shown that the RAR can be directly phosphlorated by PKA at specific serine residues (Rochette-Egly et al., 1995; Santos and Kim, 2010). Additionally, RA-induced increases in cell survival have been shown to be abolished in the presence a PKA inhibitor (Kholodenko et al., 2007), again suggesting that RA can operate through PKA to elicit some of its effects. Taken together these data suggest that RA may have the capacity to modulate the cyclic AMP signaling pathway through the modulation of PKA via an as yet undetermined mechanism. Thus, RA may be able to elicit some of its nongenomic effects through modification of this signaling pathway.

In addition to modifying the activity of PKA, recent studies have shown that RA can also modulate the activity of the PLC pathway. The PLC pathway is known to be involved in, among other things, the extracellular signal-regulated kinase pathway as well as intracellular calcium release. Work in cell culture has shown that RA exposure results in a decrease in PLC activity, suggesting that RA can inhibit the coupling of PLC to its targets (Geny et al., 1991; Jetten and

Shirley, 1985). In some studies this work has shown that the expression of PLC remained unchanged after RA exposure (Jetten and Shirley, 1985), suggesting that RA may modulate the enzymatic activity of PLC directly, rather than modify its expression. Currently, however, the details of this mechanism remain unclear. These studies add support to the growing body of literature which suggests that RA may operate independently from the identified retinoid receptors and independently of transcriptional activation. In particular, these studies suggest that RA may have the capacity to modulate PLC activity to elicit its effects.

III. Modulation of Electrophysiological Properties of Neurons

While RA may appear to modulate discrete signaling cascades within a cell (ie: specific intracellular signaling pathways), a growing body of literature also highlights the ability for RA to elicit changes in the electrophysiological properties of neurons. Such modification has the capacity to elicit changes in numerous intracellular signaling pathways and could potentially elicit widespread effects within cells. For example, work in rat has shown that treatment with RA can lead to increased spinal cord sensitization, leading the authors to suggest that RA may have altered the electrophysiological properties of neurons (Romero-Sandoval et al., 2006). Perhaps more directly, RA has been shown to cause the appearance of inward potassium currents in human cell culture lines (Arcangeli et al., 1998; Tonini et al., 1999). This RA-induced appearance of ion channels resulted in a hyperpolarization of the resting membrane potential, showing that RA has the capacity to alter the electrical properties of neurons. Other work has shown that RA can decrease sodium currents within minutes of application, leading to a change in the firing pattern of cells (Xiao et al., 1998). Importantly, this work has shown the change in sodium currents to be the result of modification of the channel gating kinetics, rather than the channel expression. Thus these data strongly argue against a "classical" transcriptional activation

role of RA and instead suggest that RA can interact with some ion channels to elicit electrophysiological changes. Similarly, application of RA has been shown to rapidly decrease the electrical coupling of some neurons (Zhang and McMahon, 2000), once again suggesting a nonclassical interaction of RA with specific channels. Lastly, the spontaneous synaptic currents of *Xenopus* neuromuscular junctions can be rapidly increased within minutes after acute RA exposure (Liao et al., 2004). This work clearly implicates RA's ability to elicit rapid electrophysiological changes in a wide array of model systems. The rapid onset of effects typically seen supports the notion that RA may operate through a nongenomic pathway. Such changes to the electrophysiological properties of cells may elicit numerous downstream effects in multiple signaling pathways, leading to widespread physiological changes within cells. Interestingly, while the electrical activity of neurons has been well known to play a critical role in neuronal development (for review see Crair (1999)) and is thought to play a role during neuronal injury and repair in adult nervous tissue (McClellan et al., 2008), no studies to date have examined whether RA may alter the electrophysiological properties of regenerating neurons.

IV. Modulation of Intracellular Calcium

Recent work has shown that RA may have the capacity to alter intracellular calcium concentrations ($[Ca]_i$) through modification of calcium fluxes (calcium influx or release from intracellular stores), as well as modify the expression of proteins that respond to changes in $[Ca]_i$. First, referring to RA's ability to modify $[Ca]_i$ through calcium influx or intracellular store release, substantial work has shown that exposure to RA can rapidly increase $[Ca]_i$ in both human (Gao et al., 1998; Launay et al., 2003) and rat (Short et al., 1991) cultured cell lines. Unfortunately, the work cited here focused on cells which were actively differentiating as a

result of RA exposure, and thus RA's effects on $[Ca]_i$ modulation in mature neurons remain unclear. Second, referring to RA's ability to modulate calcium binding proteins, work has shown that exposure to RA causes a dramatic upregulation of calcium/calmodulin protein kinase α (CaMKα) (Lawson et al., 1999; Chen and Napoli, 2008a) and an increase in the expression of S100 proteins (Wu et al., 2004; Shyu et al., 2003). Both CaMKα and S100 proteins are known to bind to calcium and thus may mediate the effects brought about by changes in $[Ca]_i$. Cumulatively, this work shows that exposure to RA may alter $[Ca]_i$ levels as well as modulate the response to calcium signals via changes in the expression of calcium binding proteins. While the exact role of RA (if any) in regulating $[Ca]_i$ remains largely unclear, these new studies suggest that RA may have the capacity to alter $[Ca]_i$ and thus represents yet another pathway through which RA may operate. Interestingly, $[Ca]_i$ levels are known to be critical to promote regeneration following injury (McClellan et al., 2008), and calcium influx (Ibarretxe et al., 2007a) as well as calcium transients (Lautermilch and Spitzer, 2000) are known to play a critical role in neurite extension. Moreover, as suggested by Mattson and Kater (1987), there appears to be an optimal range in $[Ca]_i$ required to promote regeneration, as large increases (Mattson et al., 1988b; Mattson et al., 1988a) or decreases (Connor, 1986) in $[Ca]_i$ levels adversely affect neurite outgrowth. Despite evidence that $[Ca]_i$ levels are critical to regeneration, currently no work exists investigating whether RA exposure alters $[Ca]_i$ levels in regenerating adult neurons.

In summary, this newly emerging literature has shown that RA has the capacity to modify the activity of various signaling cascades including the cyclic AMP pathway via PKA as well as the PLC pathway. Additionally, RA may modify some ion channels and thus possibly the electrical properties of neurons. Lastly, RA may also have the capacity to induce changes in $[Ca]_i$. For each effect discussed here there is an opportunity for RA to operate independently of the well-

established classical retinoid signaling pathway. Moreover, each signaling cascade discussed here can cause widespread changes within a cell. As such, transcriptional activation via the classical retinoid signaling pathway combined with the ability to modulate a number of second messenger signaling pathways vastly expands the capacity for RA to induce cellular changes. The work described above, and many other studies, illustrate a wide range of effects which RA can elicit, extending well beyond the role of a transcriptional regulator. Of particular focus for this thesis will be RA's role in a regenerative capacity, and the mechanisms by which it operates to elicit its effects. Despite being well studied in its role as a transcriptional activator in response to neuronal injury, very little is known about RA's nonclassical roles, particularly in the pathways described above, during regeneration. Thus, these pathways represent new avenues of research in elucidating the regenerative effects of RA on damaged neural tissue.

1.06 – Capacity to Regenerate in Vertebrate and Invertebrate Model Systems

In general most mammals do not readily regenerate CNS tissue after injury (for review see (Horner and Gage, 2000)). In studies typically performed in rodents, injured CNS tissue often shows an initial injury-induced response; the retraction of damaged axon stumps away from the immediate vicinity of the injury site (Horner and Gage, 2000; Luo and O'Leary, 2005; Yiu and He, 2006). Typically this process occurs hours to days after the initial injury. For a period of weeks to months after the initial injury response, mammalian CNS neurons often continue to degrade and retract further away from the injury site moving closer to the soma; a process known as neuronal degeneration or axonal degeneration (Horner and Gage, 2000; Luo and O'Leary, 2005). Similarly, in nerve or spinal cord injury paradigms the severed axons on the distal side of the damage site (which have been severed from their soma) will degrade and be broken down by macrophages in a process known as Wallerian degeneration (reviewed in (Dubovy, 2011)).

Taken together, neural regeneration (ie: the regrowth of axons past a site of injury) in the CNS of many mammals is typically nonexistent or severely impaired. As such mammals are often described as being "regeneration incapable".

While most mammals are incapable of completely regenerating damaged neural tissue, many studies have shown that the mammalian CNS has the intrinsic capacity to regenerate if given the appropriate conditions, such as permissive growth cues found in the peripheral nervous system (Aguayo et al., 1981) or various trophic and guidance factors such as neurotrophins, semaphorins (reviewed in (Yiu and He, 2006)) or, importantly, retinoic acid (Del Rincon and Scadding, 2002). Thus regeneration incapable organisms may have the ability to regenerate if exposed to the appropriate trophic and guidance factors artificially. Indeed, many vertebrates such as the newt (Oudkhir et al., 1992) axolotl (Maden, 2008) and various amphibians (Maden and Hind, 2003; Oudkhir et al., 1992) which readily regenerate damaged CNS tissue after injury appear to have altered expression of various guidance and trophic factors when compared to regeneration incapable organisms. Similarly, many invertebrate organisms such as the leech (Meriaux et al., 2011), flatworm (Zhang et al., 2008) and the snail (Lukowiak et al., 2003; Syed et al., 1992; Lee and Syed, 2004) show robust regeneration from neural damage, often leading to complete recovery. As such many of the organisms listed above are often referred to as being "regeneration capable". In such work the injured neurons will often show the initial injury-induced response seen in mammals; the immediate retraction of injured neurons from the damage site. However the injured neurons will often regenerate, rather than degenerate, axons extending through or around a damage site for a period of days, weeks or months (depending on organism studied) following an injury. Thus it would appear that a critical factor in determining whether an organism is regeneration capable is the presence of specific guidance and growth

permitting factors, such as retinoic acid, in response to injury. Of particular interest to this thesis is the regenerative capacity of the pond snail, *Lymnaea stagnalis.*

1.07 - *Lymnaea stagnalis* as a Model System for Studying Regeneration

Utilizing a molluscan model system, the pond snail *Lymnaea stagnalis,* our lab has investigated RA's regenerative effects on neurons in cell culture. In contrast to vertebrate preparations which typically employ semi-homogeneous groups of neurons of a similar cell type, this model system allows for the use of single, identified cultured neurons, with well characterized morphology and function (Figure 1.2). The use of such identified cells allows for studies to be performed in essentially identical cells, rather than populations of generally similar cells as is the case in many vertebrate preparations. Moreover, the functions and neuronal circuitry of some *Lymnaea* neurons are known which, in some cases, allows for the simultaneous observation of electrophysiological responses in identified neurons and the resulting behavioral actions (most notably learning and memory) in a semi-isolated preparation. Thus, this model system is exceptionally well suited for characterizing morphological, molecular and electrophysiological responses to various experimental conditions. Utilizing identified neurons from the CNS of *Lymnaea,* we have previously shown that, like vertebrates, RA has the capacity to elicit a regenerative response. Of particular interest is that these studies have been performed in adult neurons which, if supplied with appropriate trophic factors, will readily regenerate. Interestingly, we have shown that RA may utilize the retinoid receptors in a non-genomic capacity to elicit growth cone turning. Thus, like vertebrates, RA appears to be involved in neural regeneration in this model system. The following section will briefly review what has been documented in our lab pertaining to RA's effects on regeneration and highlight areas requiring further study that have been investigated within this thesis.

Figure 1.2. Isolated CNS of *Lymnaea stagnalis*. Arrow indicates the soma (cell body) of an identified neuron, referred to as the Right pedal dorsal 1 (RPeD1). This dopaminergic neuron has virtually identical electrophysiological properties, morphology, synaptic connections and location within the CNS across different individuals. Scale bar: 0.1mm.

1.08 – RA-induced Regeneration in *Lymnaea stagnalis*

Previously Dmetrichuk et al., (2006) have shown that RA can elicit the induction and enhancement of neurite outgrowth in identified cultured neurons of adult *L. stagnalis* in the absence of other trophic factors. More specifically, cultured Visceral F (VF) neurons exposed to RA (both atRA and 9*cis* RA) have been shown to extend significantly more neurites when compared to controls, thus showing RA's ability to induce a morphological regenerative response (Dmetrichuk et al., 2006a; Dmetrichuk et al., 2008). Additionally, the rate of outgrowth, as seen in the length of neurites over many days in culture was also significantly increased, showing RA's ability to enhance regenerative responses (Dmetrichuk et al., 2006a; Dmetrichuk et al., 2008). Furthermore, RA has also been shown to direct growth cone turning by inducing positive turns towards local gradients of RA (Dmetrichuk et al., 2006a; Dmetrichuk et

al., 2008). This work, while initially performed in neurites which were extending from the cell body (soma), has also been replicated in neurites isolated completely from the soma, where the neurite is transected from the cell body. This procedure allows for growth cone turning assays to be performed in the complete absence of the nucleus, and thus the absence of the transcriptional effects of RA. Surprisingly, directed growth cone turning was shown in these transected neurites, providing the first evidence that one aspect of RA's regenerative response (chemoattractive responses of growth cones to RA) may operate via a novel non-classical pathway. This initial work has clearly demonstrated that this model system is responsive to RA and that RA can elicit regenerative responses in cell culture.

Additionally, work has shown the presence of both atRA and 9*cis* RA isomers in *Lymnaea* CNS and hemolymph extracts utilizing HPLC (Dmetrichuk et al., 2008). Moreover, mRNA transcripts encoding full length sequences of the RAR (Carter, Genbank accession number: GU932671) and RXR (Carter et al., 2010) as well as the RALDH (Carter, Genbank accession number: FJ539101) have been cloned from cDNA generated from the isolated *Lymnaea* CNS. Lastly, RAR (Carter, 2011) and RXR (Carter et al., 2010) proteins have been detected with Western blotting of CNS extracts as well as with immunohistochemistry procedures from whole CNS samples and cultured neurons, respectively. Taken together, these experiments have verified the presence of both RA isomers, both retinoid receptors and RA synthesis machinery within this model system, thus validating the continued use of this animal model in future studies of RA.

Continued work examining RA-induced growth cone turning has used a transcription blocker to verify the nongenomic growth cone response to RA. This work has shown that the growth cone

turning response is not dependent upon transcription as exposure to actinomycin D (a known transcription blocker) does not impair growth cone turning, further implicating a novel mechanism for RA's directed regeneration (Farrar et al., 2009). Interestingly, incubation in cadmium, a blocker of voltage-gated calcium channels, was found to impair growth cone turning, suggesting that calcium influx may play a role in the RA-induced turning response (Farrar et al., 2009). Similar impairments were also observed in the presence of various RXR and RAR antagonists (Carter et al., 2010; Farrar et al., 2009; Rand et al., 2011), suggesting a unique role for the retinoid receptors, whereby the machinery of the classical retinoid signaling pathway may be functioning in a non-classical manner. Furthermore, immunostaining directed against the RXR and RAR has shown localization in the neurites and growth cones of cultured neurons (Carter et al., 2010; Carter, 2011). The presence of a nuclear receptor in a distal portion of a regenerating neuron adds further support to the role of retinoid receptors operating in a non-genomic fashion. Taken together, these data suggest that RA's regenerative effects may operate independently from the classical retinoid signaling pathway. These data lend further support to the notion that RA may operate through multiple novel pathways to elicit its effects, in particular during neuronal regeneration.

1.09 – Extension of Previous Studies

While the above work has very clearly documented a response to RA in a regenerative context, there are a number of questions which warrant further examination and study. First, while an initial morphological investigation of RA's effects has been performed in culture, no work exists to date examining whether RA can elicit similar morphological responses in the intact CNS of *Lymnaea,* or any other invertebrate CNS. Thus, continued *in situ* studies would offer a natural extension of the current cell culture experiments. Cell culture conditions lack the normal spatial and temporal signaling cues from other neurons, glial cells and muscle cells. Utilizing an *in situ*

approach to extend current studies presents a more natural (ie: *in vivo*-like) environment in which to assess neuronal regeneration. Additionally, the adult CNS of *Lymnaea* provides a rare opportunity to examine the regeneration of single identified "mapped" neurons, which possess a well-documented morphology within the CNS which is remarkably consistent between individuals. The use of RPeD1, a well-studied dopaminergic neuron, in particular provides an exciting opportunity to examine RA's effects specifically on a single identified dopaminergic neuron. Since aberrant RA signaling is thought to play a role in a number of neurological diseases, particularly those that affect dopaminergic neurons such as Parkinson's disease (Krezel et al., 1998), Schizophrenia (Krezel et al., 1998) and depression (Bremner and McCaffery, 2008) the work performed here may provide important insights into the role of RA in these diseases. Moreover, evidence suggests that application of RA may provide a viable therapeutic strategy in the treatment of these diseases, in part by promoting regeneration of injured or degenerating neurons (Katsuki et al., 2009; Ulusoy et al., 2011) as well as in response to neural injury(Maden, 2007; Maden and Hind, 2003). The first main aim of my thesis is to develop a nerve-crush injury model with a single dopaminergic neuron to study the effects of RA on regeneration. This work will allow for the investigation of RA's regenerative effects at the morphological level in injured dopaminergic neurons within the CNS. This will be the first time a detailed examination of RA-induced regenerative responses will be performed in identified dopaminergic neurons and thus could provide useful insight into the effects that RA mediates in an injured CNS.

Additionally, while it is known that RA is important for regeneration, and that RA has the capacity to operate through a number of non-classical mechanisms, very little is known about the mechanisms required for RA to elicit its regenerative effects. Typically it is thought that regeneration recapitulates developmental processes, and as such RA would be expected to

operate exclusively through the classical retinoid signaling pathway to influence gene expression. However, our work suggests a novel action of RA during regeneration of adult neurons, possibly through non-genomic effects of the retinoid receptors, although other pathways could also be involved. Since the electrophysiological properties of neurons are known to play a role in the direction/guidance of regenerating neurons (Ibarretxe et al., 2007a; McClellan et al., 2008) it is possible that RA elicits its effects on outgrowth by modifying the firing pattern of neurons. Since, to my knowledge, no studies have been performed examining RA's ability to alter the firing properties of regenerating adult neurons, my second aim will be to investigate, for the first time, RA's effects on the firing properties of regenerating adult neurons and if any effects occur, to investigate the mechanisms involved.

1.10 – Specific Objectives & Thesis Outline

The first objective of this thesis is to develop a single dopaminergic cell model of regeneration to investigate the morphological and molecular effects of RA on nerve-crush injury and regeneration *in situ*. This provides an opportunity to determine differences in response (if any) between CNS isolation (removal of the CNS from the animal, resulting in transection of peripheral nerves) and nerve-crush injury.

The second objective is to investigate the electrophysiological responses of identified neurons to RA. This includes a thorough assessment of the characteristics of the response, including time course, duration, dose and isomer dependency and determining if there are differences in response to CNS isolation and nerve-crush injury.

The third objective is to investigate the mechanism by which RA operates to elicit its electrophysiological effects. This involves an examination of the involvement of both classical transcriptional activation as well as various non-classical mechanisms, such as intracellular calcium levels, PKA and PLC signaling and protein synthesis, in the RA-induced changes.

Thesis Outline:

With these objectives established, the first data chapter seeks to investigate the morphological response to RA in the whole brain, with a particular focus on distinguishing responses to CNS isolation from that of CNS damage. This chapter will also investigate whether the retinoid receptors are altered in expression, both in the whole brain via Western blotting, and locally at the crush site utilizing immunohistochemistry. Second, very little work has examined the electrophysiological response to RA in mature neurons. Preliminary evidence suggests that RA increases the electrical excitability of neurons (Dmetrichuk et al., 2006a). However, a detailed investigation into any electrophysiological changes that are caused as a result of RA exposure, such as changes in firing pattern, has not been performed. The second data chapter seeks to characterize any such changes utilizing both *in situ* and *in vitro* approaches. Third, previous work described above (Carter et al., 2010; Dmetrichuk et al., 2008; Farrar et al., 2009) suggests a novel mechanism of action, possibly involving calcium, for RA to elicit growth cone turning. The final data chapter investigates whether RA elicits its electrophysiological changes through a number of non-transcriptional pathways in which RA has been known to operate. Cumulatively this work will provide, for the first time, a detailed examination of RA's regenerative effects in the whole CNS of an invertebrate as well as a characterization of a novel electrophysiological response to RA-exposure.

Chapter 2

The role of retinoic acid signaling in an identified dopaminergic neuron in response to a nerve-crush injury

2.01 – Abstract

The classical retinoid signalling pathway, which operates exclusively through transcriptional activation, has been well studied for its role in CNS development and vertebrate nerve regeneration. It has been previously shown that RA can induce a regenerative response, specifically the induction, enhancement and guidance of neurite outgrowth in cultured adult neurons from the CNS of the pond snail, *Lymnaea stagnalis*. These previous studies suggest that RA's effects may be conserved in invertebrate neurons and that RA may function in a similar regenerative capacity in the CNS of invertebrates and vertebrates. Currently, no work has examined whether RA can also elicit regenerative responses in damaged neural tissue in invertebrates *in situ*. As such, the first aim of this study was to determine whether RA plays a regenerative role in the intact molluscan CNS following a nerve-crush injury. To this end the fluorescent dye, Lucifer yellow, was injected into single identified dopaminergic neurons within the isolated CNS and neurite length was measured 24 hours after application of a nerve-crush injury. The data indicate that crushed dopaminergic neurons exhibited significantly more neurite length if exposed to RA over 24 hours. Furthermore, this increased neurite length appeared to be crush-specific, as no outgrowth was observed if the CNS did not receive a nerve-crush injury. To determine whether RA was operating through the classical retinoid signalling pathway, the next aim was to examine the expression of the retinoid receptors following a nerve-crush injury. First, utilizing Western blotting, it was found that expression of the RXR, but not the RAR, was diminished in the CNS 24 hours after multiple nerve-crush injuries. Secondly, utilizing immunohistochemistry to determine whether the RXR had localized to the site of nerve injury, it was found that expression of the RXR at the crush site remained unchanged compared to the uncrushed CNS. These studies provide the first evidence that RA, and signalling via the RXR receptor, may play a role in the response to a nerve-crush injury in the invertebrate CNS.

2.02 – Introduction

In addition to its role in development and patterning, RA is also known to play a regenerative role in response to neural damage in a number of vertebrate species. For example, increased protein levels of various retinoid receptors in response to neuronal injury have been reported in rat sciatic nerve (Zhelyaznik and Mey, 2006), rat spinal cord (Schrage et al., 2006), goldfish optic nerve (Nagashima et al., 2009), and zebrafish spinal cord (Reimer et al., 2009). Typically, this receptor upregulation has been shown to be maintained for several weeks after the injury, suggesting that RA plays an important role over the duration of the healing process. Previous research has also shown that the levels of RALDH, which facilitates the synthesis of RA are increased in response to neuronal injury (Zhelyaznik et al., 2003; Reimer et al., 2009; Nagashima et al., 2009). More recently, it has been shown that application of a RAR agonist can promote regeneration in rat spinal cord tissue (Agudo et al., 2010). This finding is particularly interesting as animals treated with the agonist were shown to have significantly increased axonal outgrowth of corticospinal tract neurons, which ultimately led to functional recovery *in vivo*. Taken together, the above studies demonstrate that in response to neural trauma, the RA signaling pathway is activated and upregulated in expression. The increase in expression levels of the retinoid receptors as well as the metabolic machinery required to produce and transport RA in numerous vertebrate injury models suggests an important role of RA during neuronal regeneration. While RA's effects have been investigated in cell culture, a regenerative role for RA in the intact CNS of invertebrates has not yet been studied.

While RA has been implicated to play an important role in regenerative responses to physical neuronal trauma, recent evidence suggests that RA may also play a role in a number of neurological diseases, particularly those that affect dopaminergic neurons, such as Parkinsons

disease (Krezel et al., 1998), schizophrenia (Krezel et al., 1998) and depression (Bremner and McCaffery, 2008). In general, it is thought that aberrant RA signaling occurs in the neurons affected in these diseases (Katsuki et al., 2009; Ulusoy et al., 2011). Consequently, a current therapeutic strategy that is underway is the targeted stimulation of the retinoid signaling pathway in dopaminergic neurons (Katsuki et al., 2009; Ulusoy et al., 2011). For example, oral administration of an RAR-selective agonist in mice has been shown to prevent the loss of dopaminergic neurons in the substantia nigra, the area of the brain acutely affected in Parkinson's disease (Katsuki et al., 2009). Similar work in a rat model of Parkinson's disease has shown that administration of RA can significantly increase the locomotor activity of impaired animals, suggesting that RA can cause the partial recovery of dopaminergic neurons in diseased rats (Ulusoy et al., 2011). Despite these (and many other) studies in the literature, the exact mechanisms underlying RA's effects on dopaminergic neurons remain unclear. Thus, while these studies highlight the RA signaling pathway as a potential therapeutic target in some neurological diseases, continued study is needed to further investigate the interactions of RA with dopaminergic neurons.

One likely factor contributing to the difficulty in determining the responses to RA is the inherent complexity of the nervous systems of the vertebrates used in these studies. As such, the use of an invertebrate animal model, with fewer neurons, offers the ability to resolve single, *identical* neurons across multiple individuals. In particular, the pond snail *Lymnaea stagnalis* provides the advantage of utilizing single identified dopaminergic neurons and, thus, allows for morphological and electrophysiological analysis of the same cell from one CNS to the next. In particular, one neuron known as Right pedal dorsal 1 (RPeD1) has been extensively studied, and its morphology (Lukowiak et al., 2003), synaptic connections (Lee and Syed, 2004; Lukowiak et

al., 2003) and capacity to regenerate (Lee and Syed, 2004; Syed et al., 1992; Lukowiak et al., 2003) have been previously documented. This neuron can be easily identified (and experimentally examined) within the CNS across multiple individuals. The ability to utilize a single identified dopaminergic neuron provides an opportunity to study the morphological, molecular and electrophysiological response to neuronal damage, and the role RA may play, not only in cell culture but in the intact, isolated CNS (*in situ*) as well as *in vivo*.

Previous work has shown that culturing neurons in the presence of 0.1μM atRA can induce regenerative responses in cultured *Lymnaea* neurons (Dmetrichuk et al., 2006a). Specifically, cultured Viseral F (VF) neurons exposed to 0.1μM atRA, in the absence of other trophic factors, were found to have significantly more neurites when compared to controls. Furthermore, the rate of growth of neurites, measured by changes in length was found to be significantly increased compared to controls. While these data demonstrate RA's ability to both induce and enhance neurite outgrowth in cultured VF neurons, no work has previously examined whether RA can have a regenerative effect on dopaminergic neurons. Given that RA is thought to play a role in neurological diseases involving dopaminergic neurons, extending these previous studies to include RA's effects on dopaminergic neurons could prove useful. Moreover, since all work pertaining to RA's regenerative effects in *Lymnaea* to date has been performed in culture, no work has investigated whether RA can elicit a regenerative response in the whole CNS. Utilizing an *in situ* approach to investigate RA's regenerative effects would incorporate endogenous growth factors and signaling molecules, as well as interactions (electrical and chemical) from other neighboring cells within the CNS. Such factors, which are absent in cell culture, may have a dramatic impact on regeneration and thus would more closely model an *in vivo* regenerative situation.

The first aim of this thesis was to generate an *in situ* nerve-crush injury model and to quantify a morphological response (if any) of the neurite processes of injured dopaminergic neurons. In this experimental paradigm I sought to determine whether RA was capable of eliciting a regenerative response (as seen by changes in the total length of neurite processes) in dopaminergic neurons which received a nerve-crush injury. To this end I iontophoretically injected the fluorescent indicator dye Lucifer Yellow into the identified dopaminergic neuron RPeD1, which has a well-studied and mapped morphology within the intact CNS (Scheibenstock et al., 2002). As such, the use of RPeD1 offers the ability to monitor morphological changes in fine neurite processes present in the pedal ganglion, as well as changes in the main process of RPeD1 which extends through the parietal and visceral ganglia. As such, I examined two sites of neurite processes. First, I examined the gross morphology of RPeD1 whereby I qualitatively examined the main process of RPeD1 and its projection at the crush site. Second, I quantitatively examined the fine neuritic processes surrounding the soma of RPeD1 in the pedal ganglion. As such, the development of this *in situ* nerve-crush model first required establishing whether CNS isolation alone would produce any significant changes to the morphology of RPeD1. I then aimed to determine whether exposure to RA during this 24 hour period after isolation would induce changes in RPeD1 morphology

2.03 - Materials and Methods

Chemicals. All chemicals were purchased from Sigma-Aldrich unless otherwise stated. *All-trans* retinoic acid stocks and Lucifer Yellow stocks were made fresh daily.

CNS Isolation and Tissue Culture. Laboratory reared *Lymnaea stagnalis* were housed in artificial pond water and fed lettuce as described previously (Dmetrichuk et al., 2006a; Dmetrichuk et al., 2008). Prior to dissection, animals were anesthetized by being submerged in pond water containing 25% Listerine®. Once anesthetized, animals were pinned out in a dissection dish, and the central ring ganglia (referred to hereafter as the CNS) were removed. The CNS was then bathed in antibiotic saline containing 225μg/ml gentamycin and was pinned out in a dissection dish and the outer sheath was removed. For experiments involving iontophoretic dye injection, the right pedal ganglion was positioned and pinned so as to expose the right pedal dorsal 1 (RPeD1) neuron. The bathing solution was then removed, and the surface of the pedal ganglion was partially dried with a Kimwipe®. Protease crystals were dissolved directly onto RPeD1 neurons. Protease treatment lasted for 30 – 45 seconds, after which the CNS was washed with antibiotic saline and was ready for Lucifer yellow injection. After injection of Lucifer yellow (see following section), CNS preparations were placed into a microcentrifuge tube containing 1ml of defined medium (L-15) and were incubated at 21°C for 22 hours.

Nerve-Crush Injury. The application of a nerve-crush injury was performed in a similar procedure to that used by Lukowiak et al. (2003). Briefly, isolated CNS preparations received a nerve-crush in the connective between the right pleural ganglion and right parietal ganglion. The application of the nerve-crush was applied using bare forceps to pinch the connective, thus

crushing RPeD1's main process at this site. This nerve-crush injury was applied immediately after Lucifer Yellow injection was complete.

Iontophoretic Dye Injection. Glass electrodes ranging from 25-30MΩ were pulled using a Kopf pipette puller (Model 730, David Kopf Instruments, California, USA). Electrodes were filled with Lucifer Yellow (4% wt/v, dissolved in 0.1% lithium chloride) and were backfilled with 0.1% lithium chloride. Identified RPeD1 somata were impaled and injected with 2.5-3.5nA of hyperpolarizing current pulses at 0.5Hz for 2 to 2.5 hours. During the injection procedure, the hyperpolarizing current pulses were periodically halted to verify that the electrode maintained a good seal with the cell membrane.

Fixation. 24 hours after isolation, CNS preparations were fixed in formalin (4% formaldehyde in 0.1M sodium phosphate buffered to pH 7.4) overnight. Fixed preparations were dehydrated in consecutively increasing EtOH washes (2x 50, 70, 90 and 100%) for 5 to 10 minutes per wash. Following the EtOH washes, preparations were cleared in a 100% DMSO wash for 5 minutes. During all wash stages preparations were gently agitated on a horizontal rocker, and after clearing, they were mounted on standard microscope slides with FluorSave Reagent (Calbiochem, MA, USA).

Microscopy. Lucifer Yellow-injected neurons were imaged using a Nikon C1-LU3 confocal laser scanning unit, emitting 428nm excitation light, attached to a Nikon eclipse E800 microscope running Nikon EZ-C1 viewer software (v3.2). A Nikon C1-DUD detector was set to detect emission light in the 536nm wavelength range. Specimens were observed under a 10x objective

and a single laser pass was used to acquire images for analysis. Outgrowth was analyzed by tracing the length of all neurites present in the pedal ganglion and taking the total process length value for each CNS preparation using Northern Eclipse software (v7.0). Initial experiments utilizing Z-stack arrays appeared to offer no significant difference in image quality or neruite process measurements, and as such only single pass images were acquired and analyzed.

Western Blotting. In order to investigate expression of both the RAR and RXR in the intact brain, 3 CNS preparations were isolated, pooled and homogenized for each time point of interest (0, 1, 3 and 24 hours after isolation). Preparations were homonogenized with a PowerGen handheld homogenizer (Fisher Scientific) in 500µl of lysis buffer consisting of 150 mM NaCl, 50 mM Tris–HCl (pH 7.5), 10 mM EDTA, 1% Trition X-100, 1 mM PMSF and 0.01% Protease Inhibitor Cocktail (Sigma-Aldrich). Immediately after homogenization, samples were centrifuged at 20,000x g at 4 °C for 30 minutes to separate cellular debris, after which the supernatant was collected and transferred into fresh tubes. 100µl of homogenate was used to determine protein concentration using the bicinchoninic acid assay (Pierce Protein Research Products). Fifteen micrograms of protein from each extract was separated on a discontinuous SDS polyacrylamide gel (12% resolving layer and 4% stacking layer). After separation, SDS gels were electroblotted at 100V for 1 hour onto a nitrocellulose membrane (BioRad). These procedures were repeated twice, resulting in a total of three independently generated pools of tissue (n=3) for each time point.

Western Blot Analysis. Immediately after electroblotting, membranes were washed for 15 minutes in PBS (pH 7.4), and then blocked in PBS containing 0.1% Tween-20 and 3% nonfat skim milk powder (wt./vol.) for 1 hour at room temperature. Membranes were placed on a

horizontal rocker table for gentle agitation during this time. After blocking, membranes were incubated with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (Invitrogen) at a concentration of 1:10,000 and either the affinity purified *Lymnaea* RXR or *Lymnaea* RAR antibody (Pacific Immunology) at a 1:2500 concentration in PBS,0.1% Tween-20 and 3% nonfat skim milk powder. Primary antibody incubations were left overnight at 4 °C with gentle horizontal shaking. Membranes were then washed 4 times for 5 minutes each time in PBS containing 0.1% Tween-20, after which they were incubated with a 1:15,000 dilution of Alexa Fluor 680 goat anti-rabbit secondary antibody (Invitrogen). The secondary incubation was performed at room temperature for 1 hour with gentle horizontal shaking. After secondary incubation, membranes were washed twice with PBS for 5 minutes, and then imaged with the Odyssey Infrared Imaging System I (LICOR Inc., NE, USA) at a wavelength of 700 nm. In all experiments these procedures were performed in triplicate. Western blots were analyzed densitometrically using ImageJ software (version 1.42q, W. Rasband, open source platform). The expression of RAR and RXR protein at each time point was calculated as a percent of GAPDH expression, used as a loading control, at the same time point.

Whole Mount Immunohistochemistry. The CNS was dissected in normal saline and then bathed in antibiotic saline containing 225μg/ml gentamycin for three five minute washes. Each CNS was pinned out in a dissection dish, and the outer sheath was removed. After desheathing, each CNS was placed in a 3ml petri dish containing defined medium with RA or EtOH and incubated at 21°C for 24 hours. Subsequently, they were placed in normal saline containing 0.5% type XIV protease for five minutes. Unless otherwise stated all following steps were performed at 4°C. Immediately after protease treatment, each CNS was washed in normal saline for three, five minute washes and then fixed with 4% paraformaldehyde overnight. Once fixed, each CNS was

washed for six, ten minute washes in PBS with 4% Triton X-100 and then was incubated with primary antibody at a concentration of 1:2,000 in PBS containing 4% Trition X-100 and 10% normal goat serum for 3 days. After 3 days each CNS was washed for six ten minute washes in PBS and then incubated in PBS with Alexa Fluor 488-conjugated goat anti-rabbit IgG (Molecular Probes) at a concentration of 1:1000 overnight. Preparations were then washed again with six ten minute washes in PBS followed by a ten minute incubation in DAPI at a concentration of 0.2μg/ml. After incubation, they were washed three times for five minutes each time in PBS and then mounted to slides with FluorSave Reagent (Calbiochem). Primary control experiments verifying the affinity of the antibody for RXR as well as secondary control experiments to test the specificity of the secondary antibody have been performed previously and found to validate the use of the RXR antibody (Carter, 2011).

Statistical Analysis. Analysis of data was performed with SigmaStat v3.5 software (SigmaStat Software Inc.). Differences between groups were determined either by performing a one-way ANOVA, followed by a Tukey-Kramer post Hoc test, or with Bonferroni-Holm corrected t-tests.

2.04 - Results

I. RA has no effect on RPeD1 morphology 24hr after CNS isolation.

In the following experiments I sought to distinguish whether exposure to RA would induce a regenerative response, either in the gross morphology of RPeD1 or in the fine neuritic processes, in the absence of a nerve-crush (an isolated CNS) or whether RA's regenerative response (if any) was contingent upon a nerve-crush injury. The isolation of the CNS from the animal requires all peripheral nerves to be severed, and a regenerative program may be initiated by this act. Importantly, a regenerative program initiated by CNS isolation may be different than a regenerative program initiated by a nerve-crush injury, and thus RA's effects on regeneration (if any) may be different under these two conditions. In these experiments, the CNS was isolated, and RPeD1 was immediately injected with Lucifer Yellow. The isolated CNS was then exposed to defined medium (DM) containing either 0.1μM atRA (n=6) or 0.001% EtOH vehicle control (n=5) for 24 hours, after which it was fixed and imaged. In order to obtain an estimate of the basal level (ie: time 0) of fine neurite processes in the pedal ganglion, a group of CNS preparations (n=8) was fixed immediately after Lucifer Yellow injection and imaged. In initial experiments a second group (n=13) was injected, incubated in DM for one hour and then fixed. This treatment, incorporating an additional hour for diffusion of the dye, was performed in an effort to ensure that the dye had sufficient time to fully diffuse throughout the entire neuron. In these experiments, no significant difference was found between neurons which were injected and then immediately fixed or those which were given an additional hour before fixation (t-test, data not shown). These data suggest that providing additional diffusion time of one hour does not enhance dye filling. Thus, neurons which were filled and immediately fixed were used as an estimate to determine the basal level of fine processes present on RPeD1 in the pedal ganglion. Isolated CNS preparations exposed to 0.1μM RA for 24 hours after isolation did not appear to show any dramatic changes in neuritic morphology when compared to EtOH controls or

preparations that were acutely fixed (Figure 2.1). In all cases, the gross morphology of RPeD1's main projection appeared to be consistent between groups. When measuring the total length of the neurite processes of RPeD1 in the pedal ganglion, no significant difference was found between conditions (Figure 2.2). These data suggest that the act of isolating the CNS from the animal does not significantly alter the gross morphology or the fine neurite processes of RPeD1 within the first 24 hours after isolation. More importantly, these data suggest that in the absence of a nerve-crush injury, exposure to RA for 24 hours did not affect RPeD1 morphology. In other words, transecting nerves resulted in no detectable morphological response in the presence or absence of RA over the first 24 hours. Therefore, the isolation of the CNS alone may not trigger a regenerative response.

II. RA enhances neurite morphology of RPeD1 following nerve-crush injury.

In order to determine whether RA can elicit morphological changes in nerve-crushed neurons I first sought to develop a procedure whereby the main process of RPeD1 could be damaged in the intact, isolated CNS. As shown in Figure 2.3, RPeD1 neurons which received a nerve-crush injury did not show Lucifer Yellow labeling beyond the crush point, suggesting that the application of the crush was successful and complete.

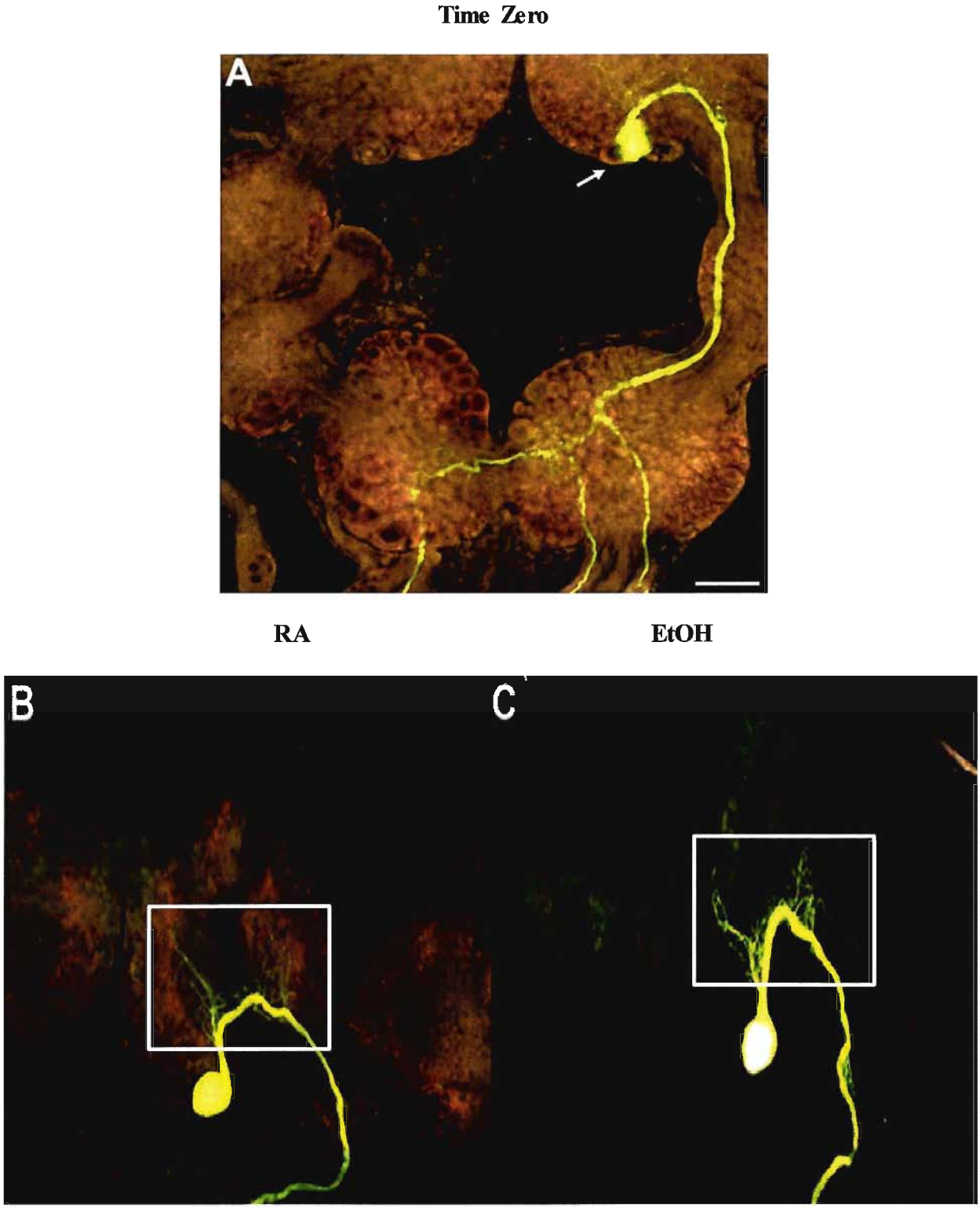


Figure 2.1. Exposure to RA does not dramatically alter the gross morphology of RPeD1 neurons in the isolated CNS in the absence of nerve-crush injury. Whole CNS images of Lucifer Yellow filled RPeD1 neurons either acutely fixed immediately after injection (A) or exposed to 0.1μM RA (B) or 0.001% EtOH (C) for 24 hours. The length of RPeD1 processes in the pedal ganglia, as shown in white boxes, was measured in subsequent analysis. Scale Bar: 150μm.

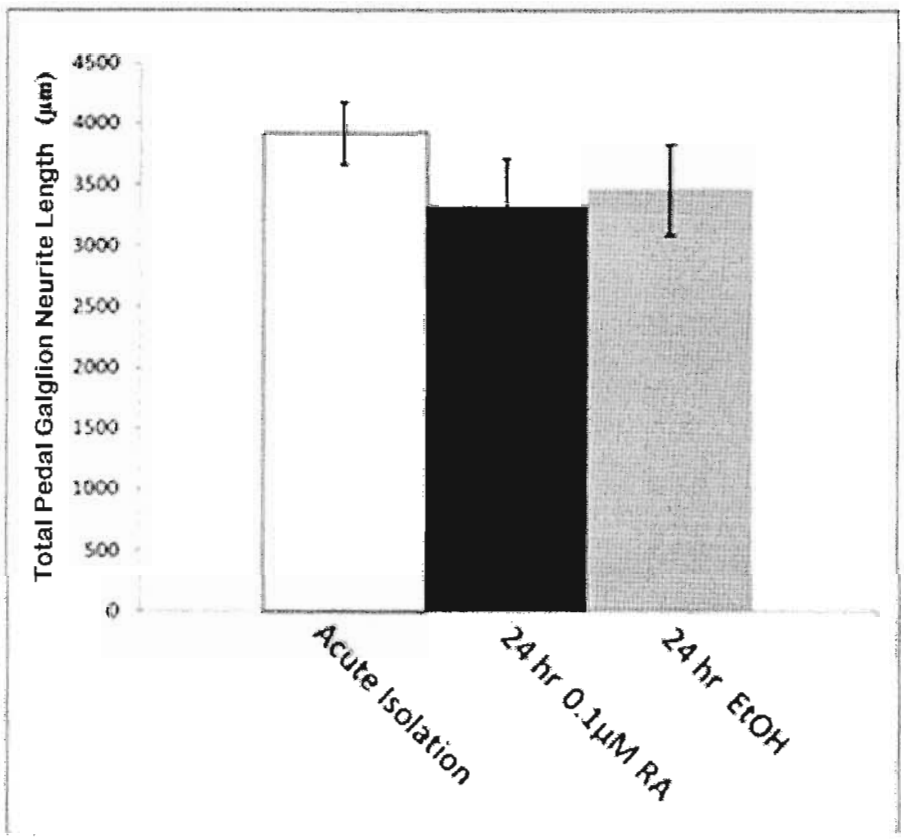


Figure 2.2. Exposure of the isolated CNS to RA for 24 hours does not alter the fine RPeD1 processes in the absence of a nerve-crush injury. The total length of fine neurites of RPeD1 measured in the pedal ganglion was not significantly different when the CNS was exposed to RA (n=6) or EtOH (n=5) for 24 hours or acutely isolated (n=8).

Having shown that application of a nerve-crush injury can completely disrupt the main axon of RPeD1, I next sought to determine whether the application of the crush injury alone would alter the neuritic morphology of RPeD1 over the first 24 hours. In these experiments I isolated CNS preparations and filled RPeD1 neurons with Lucifer Yellow. Immediately after dye filling, the preparations were nerve-crushed and incubated in DM for 1, 3 or 24 hours, at which point they were fixed and imaged. These time points were chosen in an effort to investigate the immediate (1 hour), short term (3 hour) as well as long term (24 hour) responses to an injury condition. In an effort to ensure the consistency of the nerve-crush injury, preparations which showed retraction of RPeD1's main axon out of the right pleural ganglion were excluded from further study. In these cases it was thought that application of the nerve-crush injury may have been excessive and, thus, represented a substantially more severe injury condition. Longer time points (48 & 72 hours) were also initially attempted, but proved unsuccessful, owing to a high incidence of bacterial infection.

At all time points no regeneration across the crush site appeared to occur (Figure 2.4). While no literature exists investigating regeneration of RPeD1 over 24 hours, these data are consistent with work utilizing other model systems, as recovery at lesion sites in CNS tissue occurs days to weeks after initial injury (Agudo et al., 2010; Nagashima et al., 2009). However, the nerve-crush injury decreased the total length of fine processes of RPeD1 in the pedal ganglion (Figure 2.5). More specifically, there was a time-dependent decrease in the total length of RPeD1's fine processes after application of a nerve-crush injury. This decrease was significant when CNS preparations were examined 1 hour (n=6) and 24 hours (n=6) after nerve-crush injury. Similar results have been found in previous studies utilizing rat optic nerve crushes (Koch et al., 2010; Knoferle et al., 2010) and show that, like invertebrates, nerve-crush injury resulted in the

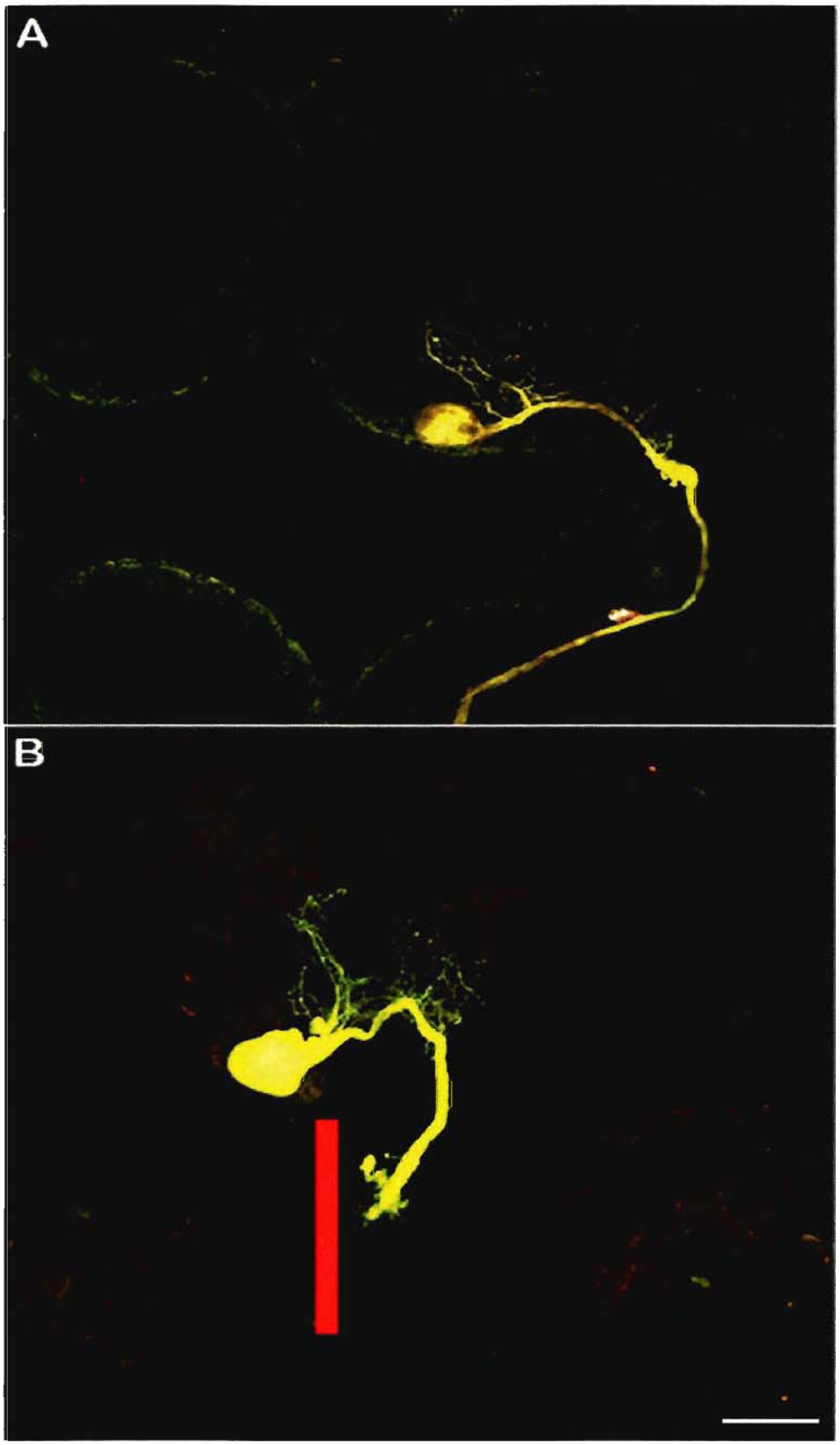


Figure 2.3. Representative examples of Lucifer yellow-filled RPeD1 neurons. CNS without (A) and with (B) a nerve crush injury. Red line indicates location of crush site. Scale bar: 150μm.

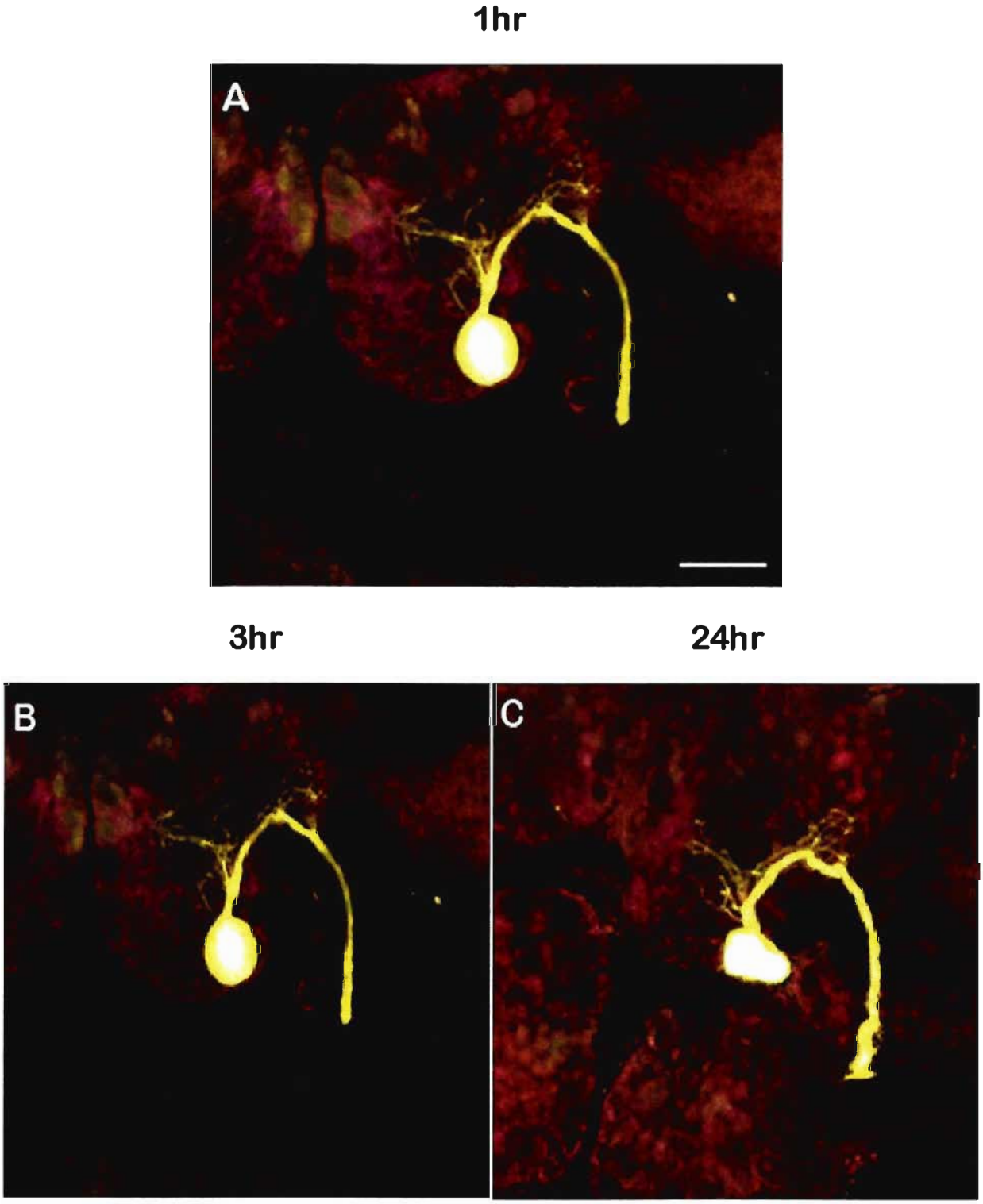


Figure 2.4. Lucifer yellow filled RPeD1 neurons exposed to a nerve-crush for up to 24 hours. Examples of RPeD1 neurons (A) 1, (B) 3 or (C) 24 hours after application of a nerve-crush injury. No regeneration across the crush site is observed in all groups. Scale bar: 150μm.

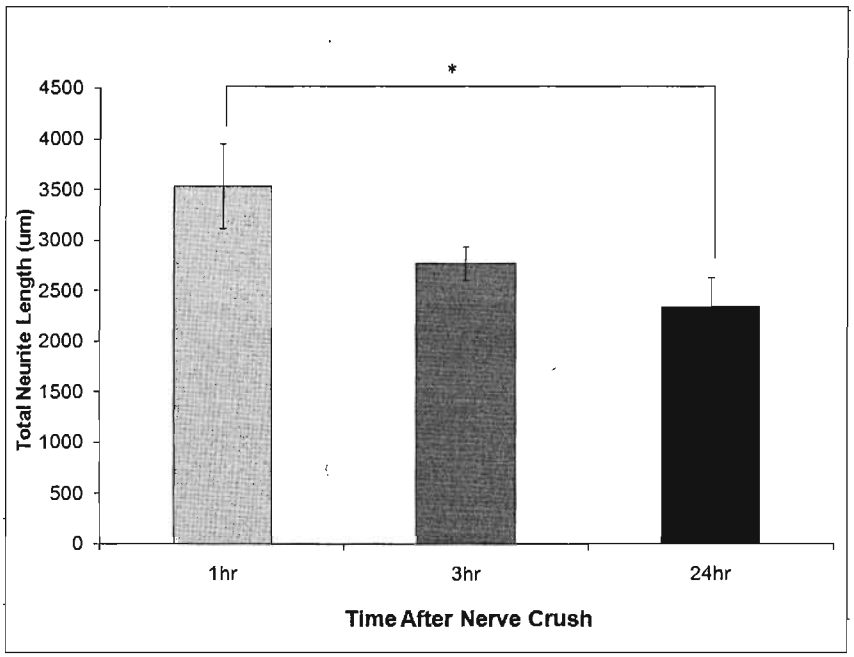


Figure 2.5. Fine RPeD1 processes in the pedal ganglion degenerate in response to a nerve-crush injury. Total neurite length of RPeD1 neurons measured within the pedal ganglion. Neurite length is significantly reduced 24 hours after a nerve-crush injury (n=6) when compared to 1hour after a nerve-crush injury (n=6).

degeneration of damaged neurons. In particular, the work shown here suggests that dopaminergic neurons degenerate in response to a nerve-crush injury over the first 24 hours.

Having developed an experimental setup wherein completely crushing RPeD1's main process causes significant degeneration of small neurites, I next sought to determine whether RA may affect this response to this nerve-crush injury. CNS preparations with a nerve-crush injury were exposed to 0.1μM RA or 0.001% EtOH (vehicle control) for 24 hours, and the gross morphology and fine neurite processes of RPeD1 were analyzed. As in all crushed preparations, the main axon of RPeD1 was found to retract slightly into the right pleural ganglion (data not shown). Interestingly, there were a small number of cases in which regeneration was observed at the nerve crush site following exposure to both RA and EtOH. As shown in Figure 2.6, 33% (4/12) of EtOH-exposed and 57% (4/7) RA-exposed preparations showed clear neurite processes extending from the damaged axon stump of RPeD1. These neurite processes had grown towards the crush site, the connective between the right pleural and right parietal ganglia. While the neurite processes clearly had extended from the axon stump, the tips of the extending neurites were not entirely resolvable (Figure 2.6, insets A & B). Thus, no quantitative analysis on neurite length was performed. While not significantly different, the trend suggested that a higher percentage of RA-exposed CNSs showed this regenerative response at the crush site, although an increased number of replicates would be required in future studies to determine if this is a significant effect of RA.

When examining the fine processes of RPeD1, exposing the CNS to EtOH (control) for 24 hours showed a significant decrease in total neurite length after nerve-crush injury (as expected from

previous data), suggesting that the presence of EtOH did not prevent the crush-induced degeneration. However, exposure to RA for 24 hours appeared to show an increase in total neurite length compared to EtOH controls (Figure 2.7; A & B). When the total length of processes was measured, an increase was indeed found in the nerve-crushed CNS exposed to 0.1μM RA for 24 hours (n=7) when compared to EtOH controls (n=12) (Figure 2.8). Interestingly the RA-exposed CNS (n=7) was not significantly different from the acutely dissected CNS (n=8). These data suggest that RA may have either enhanced regeneration following the initial crush-induced degeneration, or that RA may have served a neuroprotective role, preventing the initial crush-induced degenerative response. Unfortunately, at this time, further experiments to fully elucidate whether RA was serving a regenerative or protective role in RPeD1 could not be conducted, due to technical difficulties associated with the Lucifer Yellow injection technique. Future studies will be required to refine this regenerative model.

While the specific role RA is playing is currently unclear, these data demonstrate, for the first time, that exposure to RA can elicit a significant morphological response in an identified dopaminergic neuron within an invertebrate CNS (Figure 2.8). Importantly, this RA-induced morphological response appeared to be contingent on the application of a nerve-crush injury.

III. Nerve-crush injury results in changes in RXR, but not RAR expression.

Studies from rat (Agudo et al., 2010; Zhelyaznik et al., 2003; Zhelyaznik and Mey, 2006), goldfish (Nagashima et al., 2009) and zebra fish (Reimer et al., 2009) have shown that both the RAR and RXR receptors can be upregulated in response to neural damage in vertebrates. No work, however, has investigated whether this injury-induced activation of the retinoid receptor

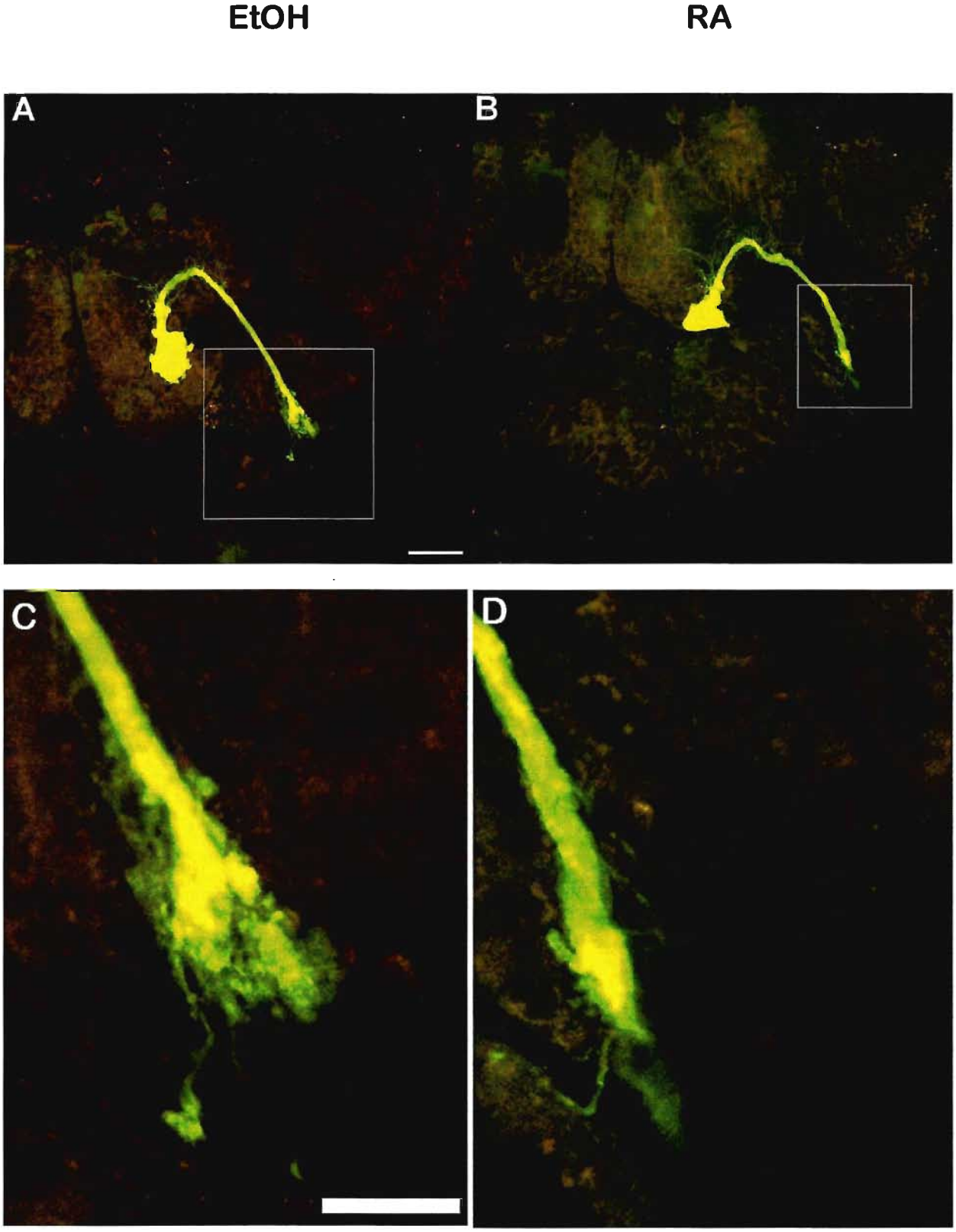


Figure 2.6. Representative examples of regenerative responses occurring at the crush site. CNS preparations exposed to (A) 0.001% EtOH or (B) 0.1μM RA for 24 hours showed regeneration at the crush site (EtOH: 4 of 12 preparations, RA 4 of 7 preparations). A & B are enlargements of the boxed areas from A and B respectively. Scale bars: A & B: 150 μm, C & D: 75 μm.

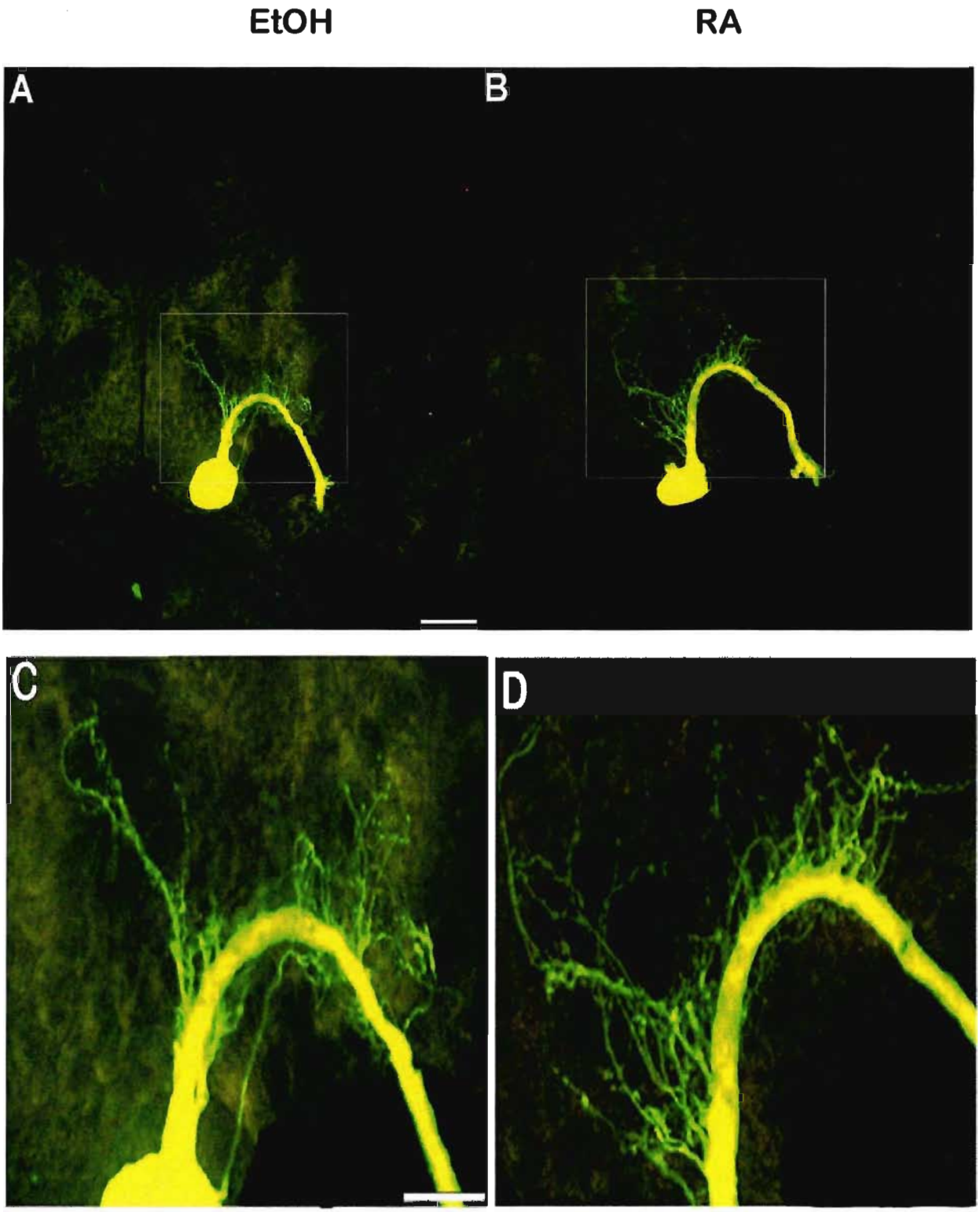


Figure 2.7. Representative examples of crushed RPeD1 neurons in the presence of RA and EtOH. Representative examples of Lucifer yellow filled RPeD1 neurons from the isolated CNS exposed to (A) 0.001% EtOH (n=12) or (B) 0.1μM RA (n=7) for 24 hours after a nerve crush injury. C & D are enlargements of the boxed areas from A and B respectively. Scale bars: A & B: 150 μm, C & D: 75 μm.

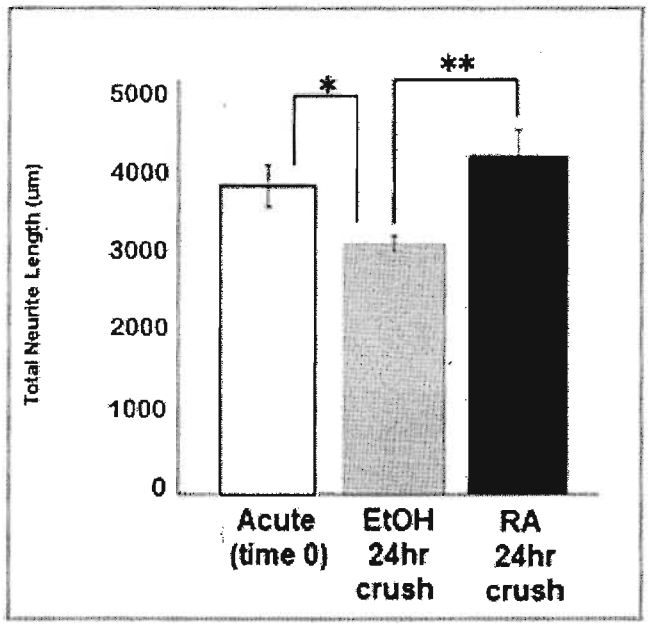


Figure 2.8. RA-exposed CNS shows significantly more total neurite length following a nerve–crush injury. Analysis of RPeD1 neurite length in the pedal ganglion. 0.1µM RA-exposed neurons (n=7) showed significantly longer neurite length when compared to vehicle control (n=12) at the same time point. 0.001% EtOH-exposed neurons showed significantly reduced neurite length compared to acutely fixed controls (n=8). (*$p<0.05$, **$p<0.01$)

pathway is conserved in our invertebrate model system or other invertebrate systems. Furthermore, given the plethora of pathways through which RA can operate, including PKA (Rochette-Egly et al., 1995; Santos and Kim, 2010), PLC (Jetten and Shirley, 1985; Haase et al., 1997) and intracellular calcium signaling (Gao et al., 1998; Launay et al., 2003), it is currently unclear whether the response to RA observed following a nerve-crush injury operates through the activation of the classical retinoid receptor pathway or possibly via an alternative, nontranscriptional mechanism. Having developed a nerve-crush injury paradigm in which a degenerating dopaminergic neuron shows an RA-dependent regenerative response, I next sought to determine (a) if expression levels of the retinoid receptors respond to the nerve-crush injury, and (b) if exogenous application of RA affects the expression levels.

i. RXR expression is downregulated with increasing levels of nerve injury over 24 hours.

In these experiments, the CNS was isolated and exposed to one of three conditions: 1) no nerve-crush injury, in which the CNS was merely dissected, 2) a "single nerve-crush" in which a single nerve-crush was applied in the same location as described in the previous section, between the pleural and parietal ganglia, and 3) a "multi-nerve-crush" in which each connective within the CNS was crushed. The latter condition was performed in order to amplify any subtle molecular changes that might occur in response to a single nerve-crush injury. Once isolated, the CNS was either snap frozen on liquid nitrogen, to establish basal levels of receptor expression, or incubated in DM for 1, 3 or 24 hours as performed in the previous section, and then subsequently frozen. In order to generate sufficient total protein for western blot detection, and to minimize inter-individual variation, each time point used 3 CNS preparations which were combined as one sample for analysis. Western blotting was run in triplicate from 3 separate samples (3 groups of 3

preparations per group), and RAR or RXR expression was calculated as a percent of the loading control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a commonly used "housekeeping protein" thought to be ubiquitously expressed (Carter, thesis). Representative examples of Western blots showing RAR and RXR expression over all conditions are shown in Figure 2.9.

Overall, the expression of the RAR was found to remain relatively stable over each time point, regardless of the level of nerve-crush injury (Figure 2.10, Ai to Aiii). RAR expression appeared to vary by no more than ~35% between time points. In contrast, however, the RXR was found to be differentially regulated in various conditions (Figure 2.10, Bi to Biii). The RXR appeared to be upregulated over the first 24 hours in a no-crush (isolation only) condition (Figure 2.10, Bi). These data show a 63% increase in RXR expression between basal level expression (time 0) and 24 hours after isolation. While not significant ($p=0.212$), these data suggest a trend toward upregulation of the RXR in response to CNS isolation, though additional replicates would be required in order to verify this claim. Interestingly, RXR expression showed a significant decrease ($p<0.013$) in the multiple nerve-crush condition to levels approximately 38% (ie: a 62% reduction) of basal levels 24 hours after nerve-crush injury. Overall, these data suggest that RAR expression remains largely unchanged during CNS isolation and varying levels of nerve-crush injury, but that the RXR may show a modest trend of upregulation in a non-injured CNS isolation condition. This upregulation is reversed, showing a significant downregulation in a multi-crush nerve injury condition. Taken together, these observations suggest that the RXR, but not the RAR, may be involved in a molecular response to a nerve-crush injury.

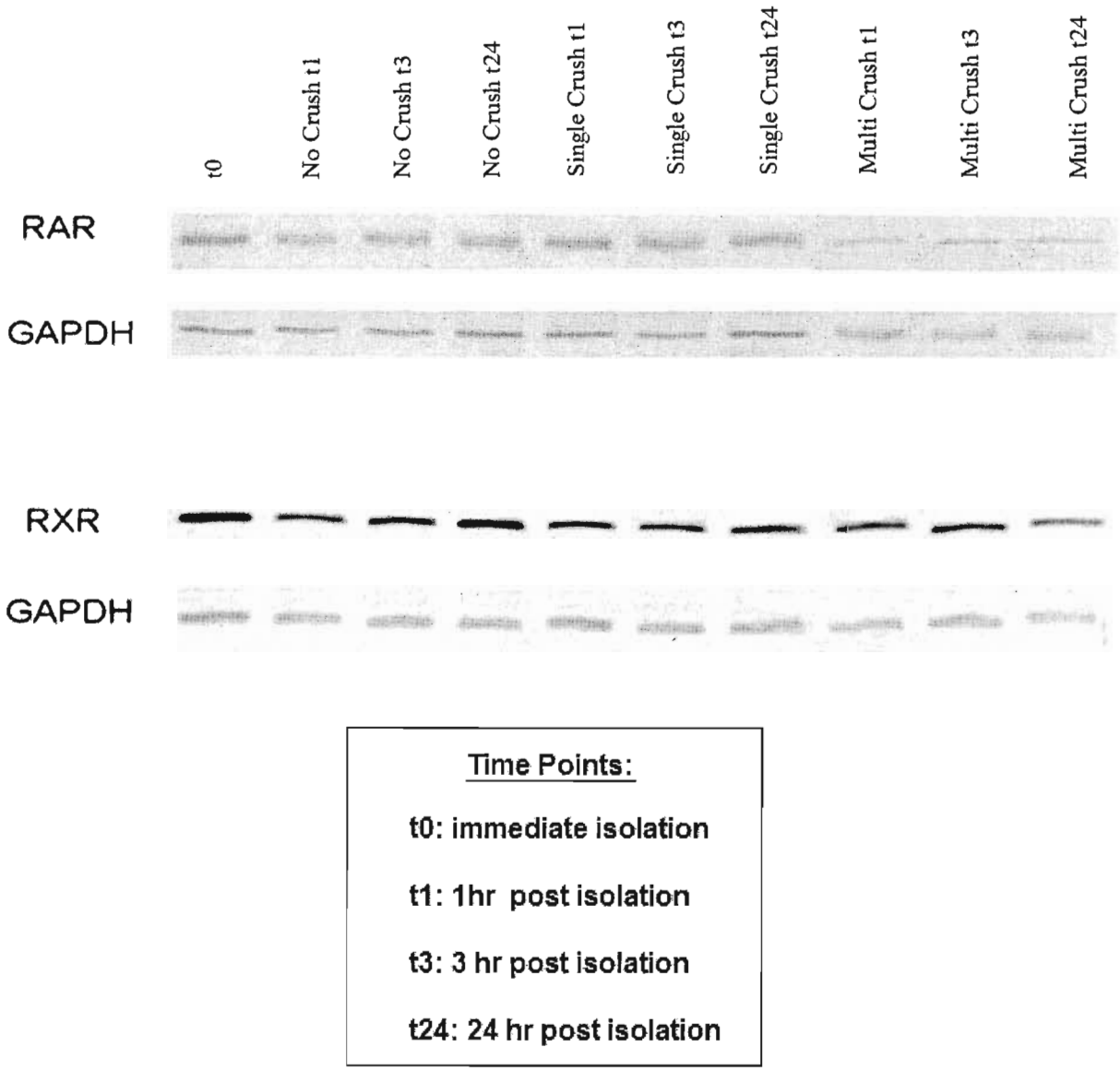


Figure 2.9. RAR and RXR expression 1, 3 and 24 hours after application of varying levels of nerve crush injury. Representative results from one of three independently performed Western blots. RAR (upper) and RXR (lower) expression was calculated densitometrically as a ratio of GAPDH expression for each western blot respectively. Note: crush conditions received nerve-crush immediately after CNS isolation. n=3 in all conditions. Full Western blots are shown in Appendix 1.01-A.

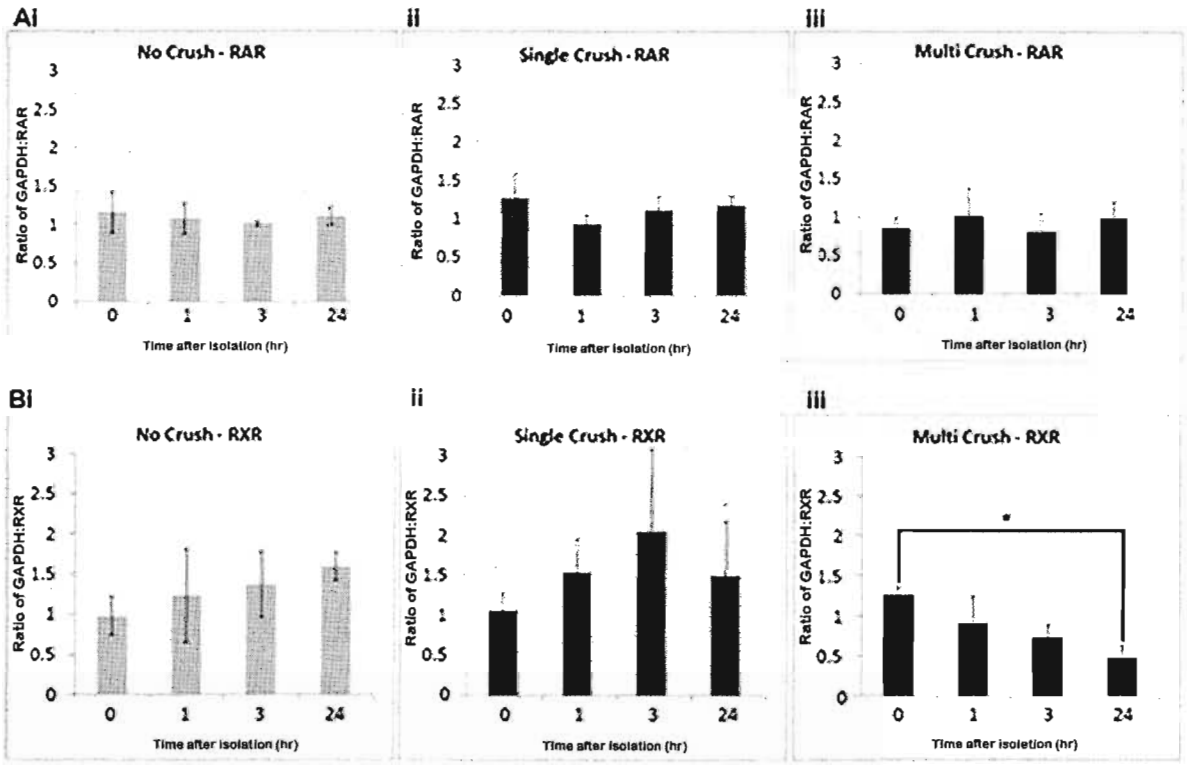


Figure 2.10. RXR expression is altered following CNS isolation and varying levels of nerve-crush injury. A: RAR expression calculated as a ratio of GAPDH expression (set at 1). RAR expression appears unaltered during the first 24 hours of CNS isolation (i) and during both a single (ii) and multi-crush (ii) injury. B: RXR expression remains unchanged in no crush (i) and single crush injury (ii) conditions. During a multiple crush injury (iii) a downregulation is shown at the 24 hour time point. (t-test, n=3 all conditions, *= $p<0.05$).

ii. Retinoid receptor expression during a multi-crush nerve injury and subsequent exposure to RA.

Having shown that the RXR, but not the RAR, is downregulated during a multi-crush nerve injury condition, I next sought to determine whether exposure to RA could modulate the expression levels of the retinoid receptors during this injury condition. Both the RAR and RXR have been previously shown to be autoregulated in other vertebrate models (Ribot et al., 2004; Duprez et al., 1996) and thus exposure to RA during a multi-crush nerve injury may alter the expression levels of either receptor, possibly promoting a regenerative response. To this end, I isolated and exposed the CNS to a multi-crush nerve injury, followed by incubation in either 1μM atRA or 0.01%EtOH (as a vehicle control). In order to determine if any RA-induced changes in expression were dependent on an active regenerative program, I performed identical experiments in parallel with uncrushed CNSs. Representative examples of RAR and RXR expression over all conditions are shown in Figure 2.11. Three independent series of experiments were performed and densitometric measurements were averaged, as performed previously.

Overall, both the RAR as well as the RXR expression levels, regardless of treatment, were found to remain largely unchanged. First with respect to the RAR, no significant differences were found either between RA and EtOH treatments over time or between the crushed and uncrushed CNS (Figure 2.12). These data suggest that RA exposure does not alter the expression of the RAR in the presence or absence of a nerve-crush injury. These data were in agreement with my previous Western blotting results, indicating that the RAR remains largely unchanged during the first 24 hours of a nerve-crush injury. These data also suggest that RA exposure does not elicit dramatic increases in the RAR expression levels within the first 24 hours.

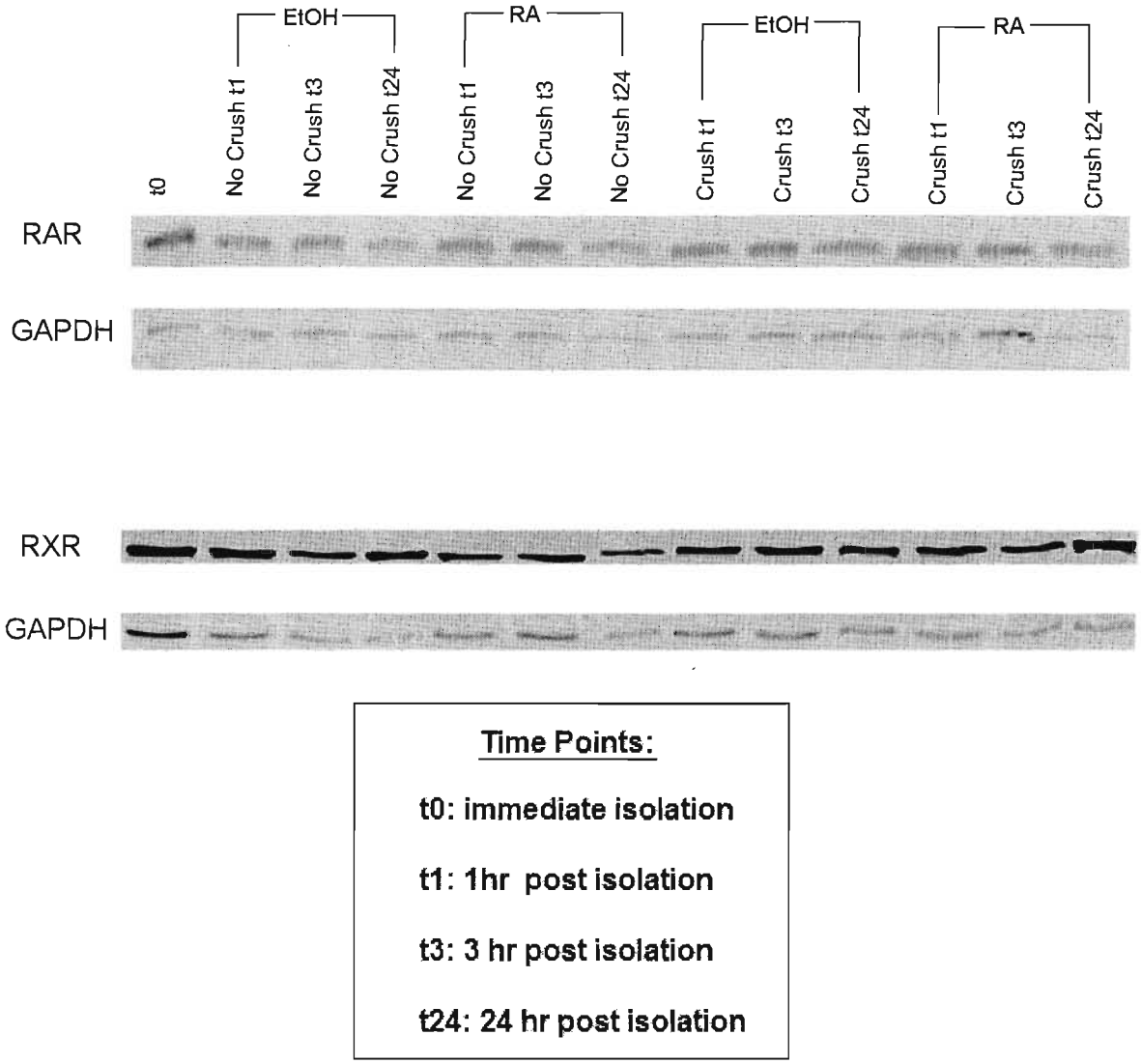


Figure 2.11. RAR and RXR expression after application of nerve-crush injury in the presence of RA or EtOH. Representative results from one of three independently performed Western blots. RAR (upper) and RXR (lower) expression was calculated densitometrically as a ratio of GAPDH expression for each western blot respectively. Note: crush conditions received nerve-crush immediately after CNS isolation. n=3 in all conditions. Full Western blots are shown in Appendix 1.01-B.

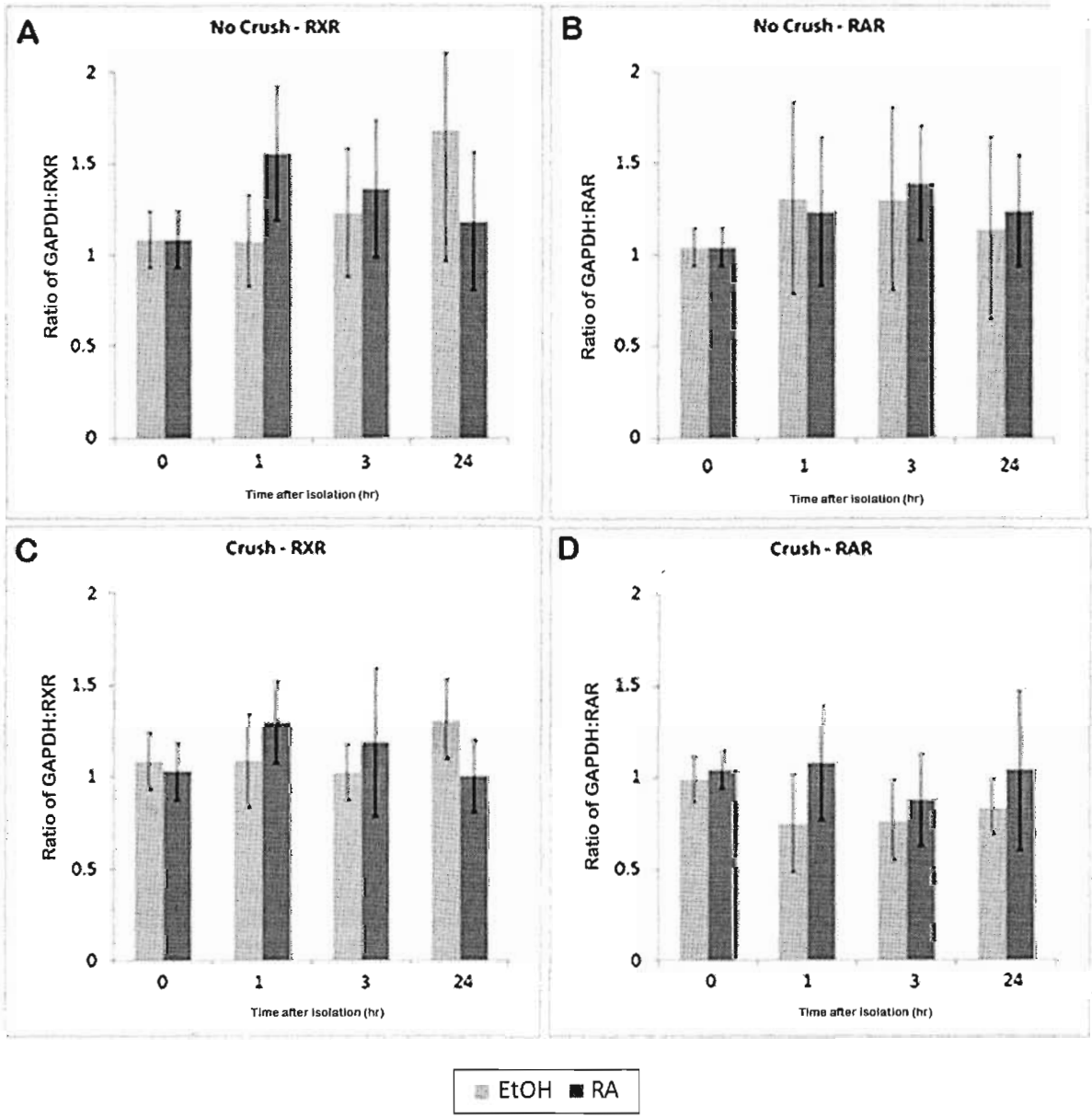


Figure 2.12. Exposure to RA during a nerve-crush injury does not alter the expression of the RAR or RXR. The expression of the RAR (A & B) and RXR (C & D) did not change during a nerve-crush injury and subsequent RA exposure. RA exposure did not alter the expression of the RAR or RXR within the first 24 hours after CNS isolation in the absence of a nerve-crush injury (A & C) or with the application of a nerve crush injury (B & D). In all cases no significant differences were found between RA and EtOH-exposed CNSs at any time point in any crush condition. n=3 in all conditions.

Interestingly, similar results showing no significant change in expression were found for the RXR in both uncrushed CNS and CNS exposed to a multi-crush nerve injury (Figure 2.12). As shown in Figure 2.12, no statistical difference was found when comparing RA to EtOH-exposed preparations in either crush condition at any time point. Importantly, EtOH-exposed preparations which were exposed to a multi-crush injury did not show an injury-induced decrease in RXR expression, as would have been expected from the previous section (as in Figure 2.10, Biii). Since a significant decrease in EtOH-exposed preparations exposed to a multi-crush injury was not observed, these data suggest that EtOH exposure may induce a vehicle effect, whereby EtOH exposure alone results in alterations in RXR expression, and would thus mask any RA-induced alterations in RXR expression 24 hours after a multiple nerve-crush injury.

Since EtOH exposure has been shown in some cases to increase expression of the RXR (Kumar et al., 2010), I hypothesized that exposing the CNS to EtOH may have masked any crush-induced changes in expression. As such, I next performed a separate series of experiments whereby I exposed the crushed CNS to 0.01% EtOH or 0.01% DMSO (an alternative vehicle commonly used to dissolve RA) for 24 hours. In the absence of any vehicle, the multi-crush injury induced a 33% decrease in RXR expression compared to controls (basal expression at time 0), as in previous experiments (Figure 2.13). Interestingly however, the crushed CNS exposed to either 0.01% EtOH or 0.01% DMSO did not show this decreased RXR expression (Figure 2.13, A). Similar results were obtained when these experiments were repeated using a second series of isolated CNS preparations (data not shown). RAR expression was also run in parallel and remained largely unchanged (Figure 2.13, B), as in agreement with my previous results (Figure 2.10, Biii). Overall these data strongly suggest that the presence of the vehicles EtOH and/or DMSO prevented the crush-induced decrease in RXR expression. Since RA is not soluble

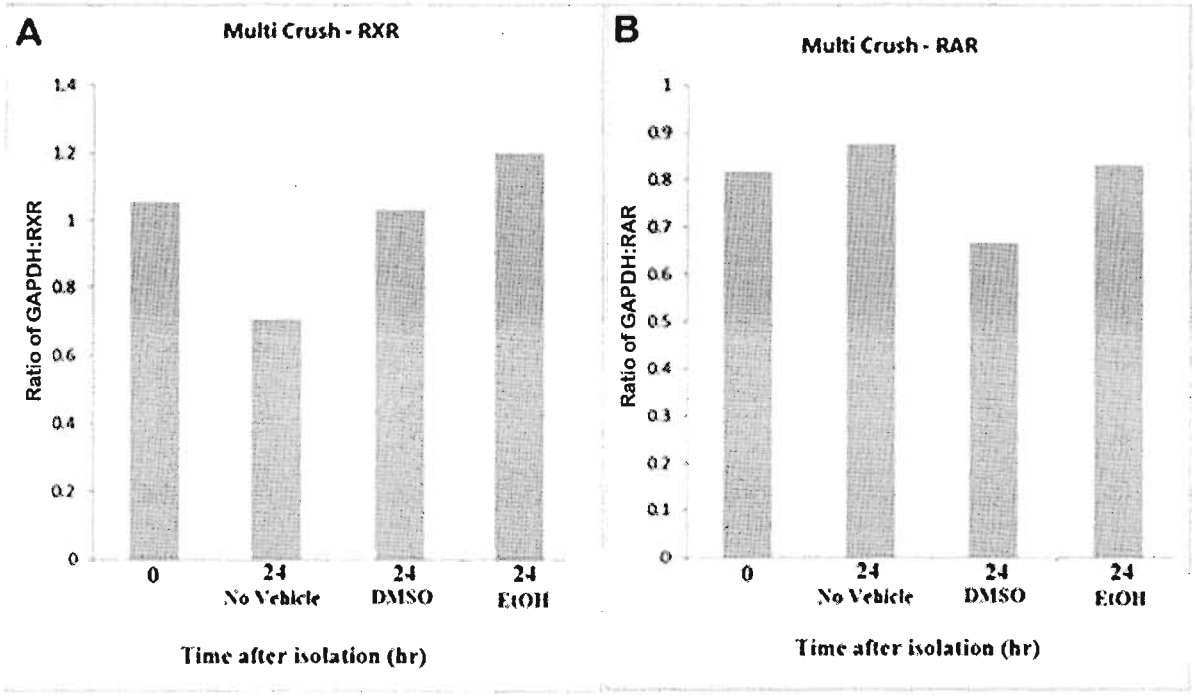


Figure 2.13. Exposure to EtOH or DMSO blocks the crush-induced decrease in RXR expression. The expression of the RXR (A) and RAR (B) in nerve-crushed CNS preparations exposed to either DMSO, EtOH or left in defined medium only (no vehicle). A: As expected, RXR expression is diminished 24 hours after the application of a nerve-crush injury in the absence of any vehicle. RXR expression does not show a crush-induced decrease in the presence of DMSO or EtOH 24 hours after a nerve-crush injury. B: RAR expression remains largely unaltered regardless of vehicle exposure. Similar results were obtained from a second set of experiments (n=2).

in water, it was not possible to continue any investigation into the effects of RA on injury-induced changes in RXR expression.

The previous work utilizing Western blotting procedures studied the response of the entire CNS to the nerve-crush injury and so did not look at localized responses. Considerable work has shown that the RXR can translocate to cellular microdomains in response to various cues. For example, the RXR has been shown to localize in some regenerating axons within the rat spinal cord after injury (Zhelyaznik and Mey, 2006). Similarly, application of plant extracts (Zeng et al., 2006) or serum (Han et al., 2009) can cause rapid translocation of the RXR out of the nucleus into cytoplasmic domains. Such observations suggest that the RXR may respond locally at specific sites of neural damage without necessarily showing widespread changes in expression.

My next aim was to utilize immunohistochemistry to investigate any localized changes in RXR expression in response to the nerve-crush injury. Of particular interest was whether the RXR localizes to the crush site within the CNS.

IV. The RXR does not appear to localize to the crush site following a nerve-crush injury.

In order to determine whether RXR expression is altered locally in response to nerve-crush injury, I first exposed the CNS to a single nerve-crush injury for 24 hours, as performed previously. That is, each CNS was again given a single crush between the pleural and parietal ganglia. Immediately after application of the nerve crush, each CNS was incubated in DM for 24 hours and subsequently was fixed. A control condition consisted of incubating the uncrushed CNS in DM for 24 hours. In order to determine basal levels of RXR expression, a third group of

preparations was isolated and immediately fixed. Preparations from all three conditions were subjected to immunohistochemistry procedures at the same time and then were imaged individually.

In preliminary experiments, RXR staining appeared ubiquitous in all areas of the CNS including all ganglia, connectives and peripheral nerve ends. While individual cell bodies on the surface of the ganglia were clearly visible, this was only the case if the soma was positioned on the dorsal most surface of the CNS. Due to their location within the ganglia, RPeD1 somata were frequently undetectable. Since I was unable to visualize RXR staining directly in RPeD1, the following experiments comprised a qualitative assessment of the overall staining intensity of the RXR across the ganglia and the nerve-crush site. As shown in Figure 2.14, the RXR expression across the entire CNS appeared largely unchanged within the first 24 hours after a nerve-crush injury. More specifically, uncrushed preparations which were acutely fixed (n=7) appeared to have fairly uniform RXR staining intensity across both the right pleural and right parietal ganglia as well as the connective joining the ganglia, which was targeted in other preparations for the application of the crush injury (Figure 2.14, asterisk). Preparations receiving multiple nerve-crush injuries (n=7) as well as preparations which were left uncrushed (n=5) for 24 hours also showed similar uniform RXR staining intensity in these areas. When comparing acutely fixed preparations to those isolated for 24 hours, there appeared to be no dramatic difference in the intensity or the distribution of RXR staining. This suggests that isolation alone for 24 hours does not dramatically alter the distribution of RXR within the CNS. Additionally, since crushed and uncrushed preparations appeared similar, with no obvious difference in staining intensity or localization, these data suggest that RXR expression is not dramatically altered at the site of a nerve-crush injury within the first 24 hours.

Since the results suggest that in the absence of exogenous RA, the RXR does not localize to the nerve-crush site, I next sought to determine if RA exposure during a nerve-crush injury would alter the localization of the RXR within the CNS. More specifically I isolated the CNS, applied a single nerve-crush as performed previously and then exposed each CNS to 1μM RA or 0.01% EtOH as a vehicle control for 24 hours. Uncrushed preparations which were exposed to RA or EtOH for 24 hours were run in parallel with these experiments to control for isolation-dependent changes. When examining RXR expression at the crush site, no substantial increase in RXR staining was observed in crushed preparations exposed to RA or EtOH for 24 hours (Figure 2.15). In both RA and EtOH-exposed groups, the crush site had a similar staining intensity and distribution to that of uncrushed group exposed to RA and EtOH for 24 hours (n=7 for all conditions). These data suggest that exposure to RA did not substantially alter expression of the RXR at the crush site within the first 24 hours after a nerve-crush injury.

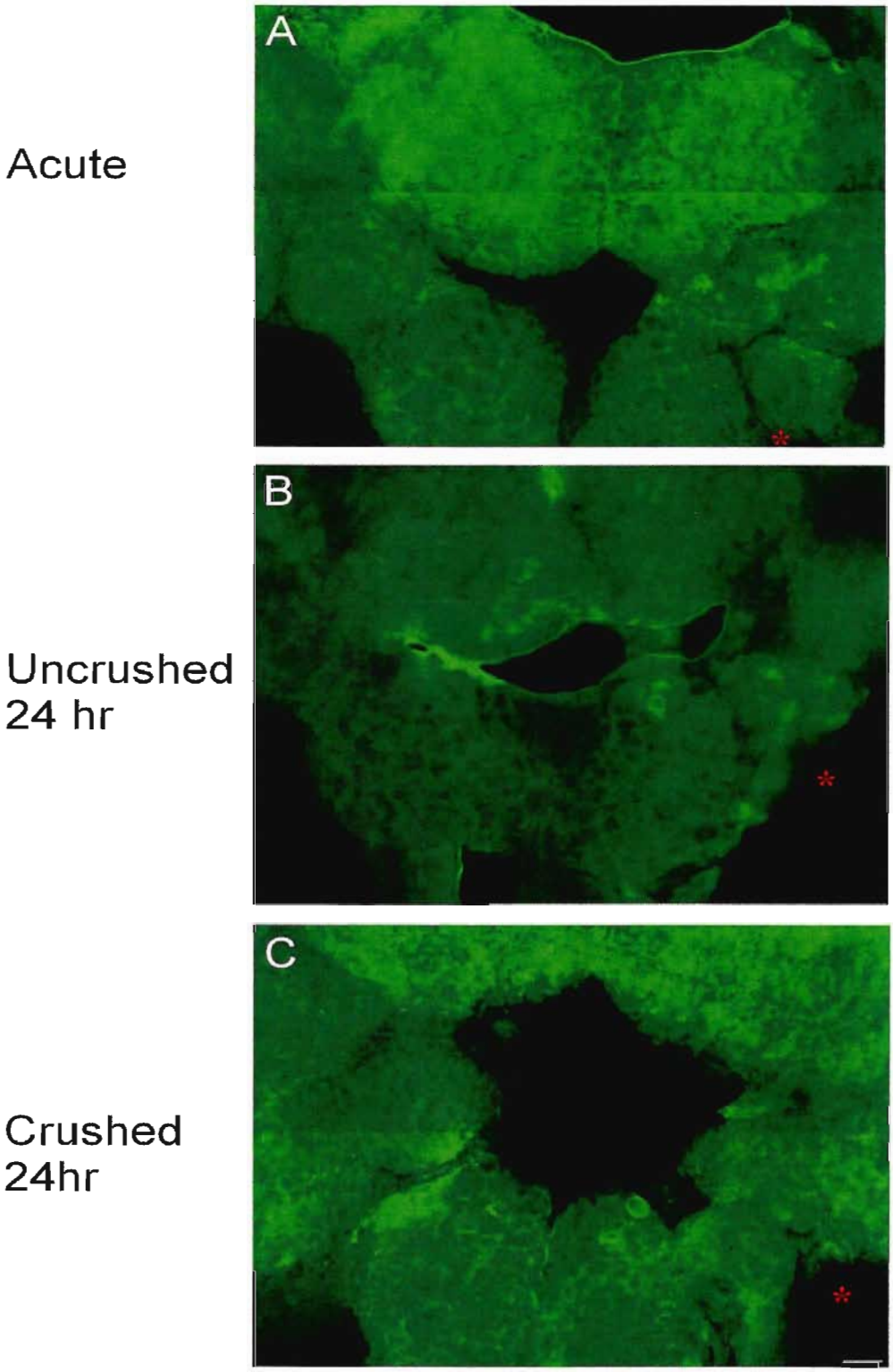


Figure 2.14. RXR expression does not appear to dramatically change in response to a nerve-crush injury within the first 24 hours. A; Uncrushed CNS immediately fixed (n=7) after isolation from animal. B; CNS which was isolated (uncrushed) for 24 hours (n=5). C; CNS which received a nerve crush injury for 24 hours (n=7). In all cases the RXR expression appeared to remain consistent across the CNS and crush site. Star indicates crush site. Note that A and C have crush sites marked for clarity; however, these are not crushed. Scale bar = 125 μm.

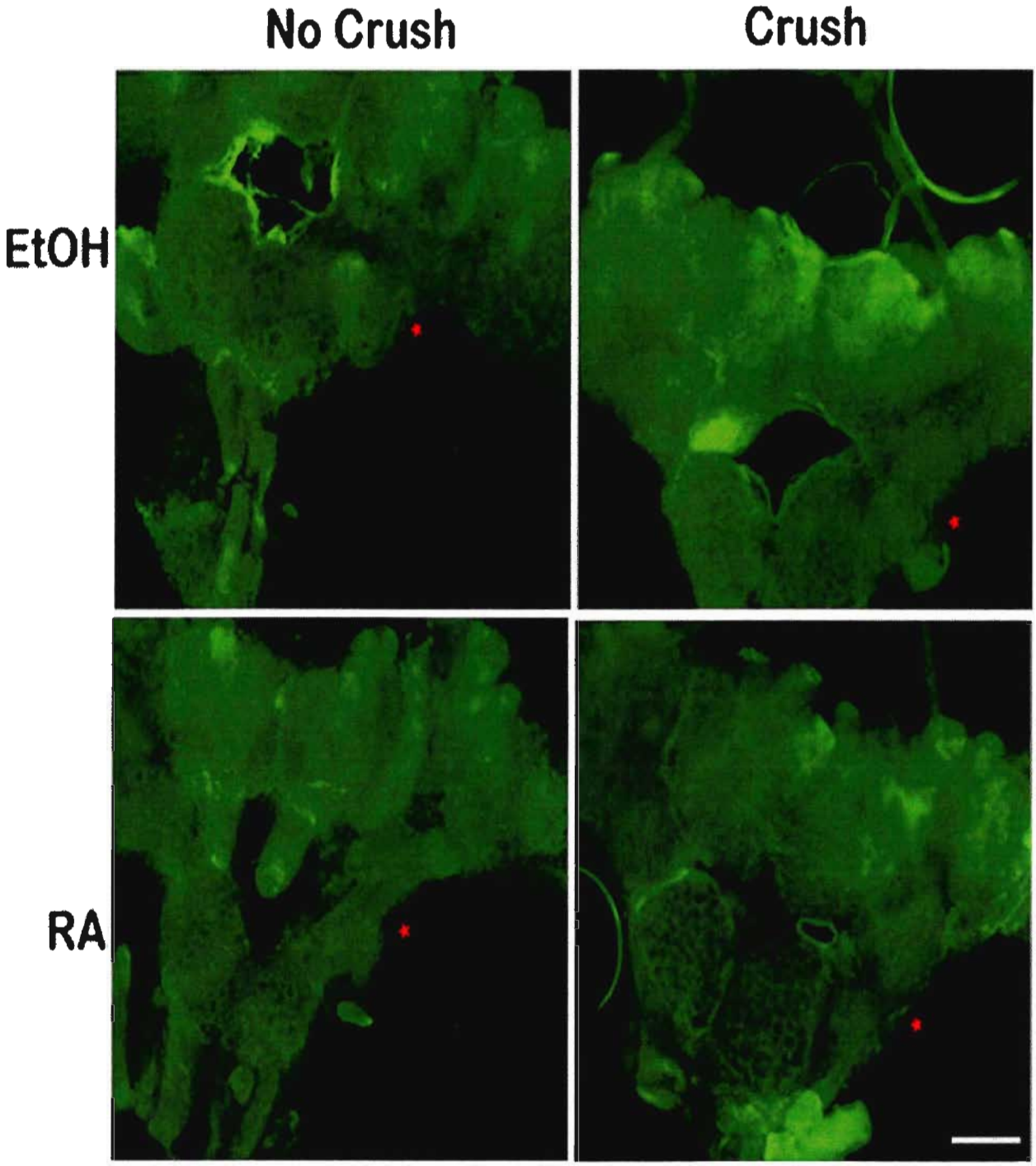


Figure 2.15. RA exposure does not appear to alter RXR expression in both uncrushed and crushed CNS within the first 24 hours. In all cases the RXR expression appears to remain consistent across the CNS and crush site. Star indicates area of crush site. Note that each uncrushed CNS has a crush site marked for clarity; however, these are uncrushed. Scale bar = 250 μm. n=7 for all conditions

2.05 – Discussion

In this study I aimed to develop a single cell assay to study the effects of RA on regeneration in identified dopaminergic neurons. I determined, for the first time, that RA was capable of producing nerve-crush dependent effects on the neuritic morphology of the dopaminergic neuron, RPeD1. Interestingly, I have showed that in the entire CNS, severe nerve-crush injury produced a significant overall decrease in RXR expression over 24 hours. No changes in RAR expression were evident from any of the nerve-crush conditions. While I was unable to fully investigate or determine the effects of RA on RXR expression during a nerve-crush injury, due to the effects produced by the vehicle alone, I was able to show no dramatic localization of the RXR to the crush site.

These data showed that direct application of RA promoted a morphological response in an identified dopaminergic neuron in the isolated CNS. More specifically, the total length of fine neurite processes of RPeD1 present in the pedal ganglion was significantly increased upon exposure to RA. Furthermore, this RA-induced morphological change was dependent upon on a nerve-crush injury. Uncrushed dopaminergic neurons appeared to be unresponsive to RA exposure, suggesting that neuronal injury, and not CNS isolation alone, was required to elicit the RA-induced morphological effects. These data are in line with previous studies which suggest that damaged neuronal tissue is responsive to RA. For example, previous work has shown that retinoid receptor agonists can promote neurite outgrowth in the injured spinal cord of rat (Agudo et al., 2010). Similarly, direct application of RA has been shown to lead to neurite outgrowth in the damaged optic nerve of the goldfish (Nagashima et al., 2009) and in newt spinal cord explants (Dmetrichuk et al., 2005). Taken together, these data show, for the first time in

Lymnaea, that RA exposure can produce a regenerative response in the isolated CNS and possibly promote outgrowth in an identified dopaminergic neuron.

Currently it is unclear whether RA is acting as a stimulator of neuritic outgrowth to increase the regeneration of processes or as a neuroprotectant to reduce degeneration in response to the nerve-crush injury (ie: reducing neuronal degeneration). First, it is possible that RA elicits new outgrowth from RPeD1 over the first 24 hours of a nerve-crush. In other words, the application of RA may be unable to prevent the degeneration that results from the nerve-crush injury, though RA may be able to induce new outgrowth from a damaged RPeD1 neuron. In this role, RA would act as a stimulant, promoting new outgrowth. Previous work lends support to this notion, showing that RA exposure can cause increased neurite outgrowth in mice (So et al., 2006) and rats (Agudo et al., 2010) which have received a nerve-crush injury for 3 and 14 days, respectively. While these studies did not look at the response to RA over the first 24 hours of application, recent evidence from *Lymnaea* has shown that cultured invertebrate neurons respond to RA with increased neurite outgrowth over the first 24 hours (Dmetrichuk et al., 2006a). Taken together, there is substantial evidence suggesting that RA can induce new outgrowth from damaged neurons, thus supporting the possibility that in this injury paradigm, RA may act as a stimulator of neuritic outgrowth.

Alternatively, a second possibility is that RA does not induce any new outgrowth, but rather preserves or maintains the existing processes, thus preventing neurodegeneration. In this role, RA acts as a neuroprotectant. While less prevalent in the literature, there is some evidence supporting this hypothesis. For example, work utilizing rat midbrain dopaminergic neurons has shown that pretreatment with a RAR agonist can protect neurons against inflammatory

degeneration (Katsuki et al., 2009). Similarly, RA can protect neurons from induced cell death in chick neurons (Ahlemeyer and Krieglstein, 2000), thus highlighting the neuroprotective capacity of RA. While future studies would be required to determine the exact role RA is playing, either possibility (outgrowth stimulation or neuroprotection) could have implications for the use of RA in the treatment of dopaminergic neurological diseases.

Interestingly, there were a small number of cases (4 of 12 and 4 of 7 for EtOH and RA-exposed CNS, respectively) in which regeneration was observed at the nerve-crush site following exposure to both RA and EtOH. Unfortunately, at the 24 hour time point only a small minority of preparations displayed such regeneration. While it is tempting to speculate that RA may facilitate this regeneration given that a higher number of RA-exposed preparations displayed crush site regeneration, additional experiments would be required to validate this, as no statistical difference was found. Continued efforts were made to extend the incubation time point beyond 24 hours, but a high prevalence of bacterial infection prevented such work from continuing.

Having established that over the first 24 hours after a nerve-crush injury the processes of RPeD1 retract, I next sought to determine if this morphological response was paralleled with a molecular response, an alteration in retinoid receptor expression. Substantial work has shown that the retinoid receptors are upregulated in response to neural trauma (Agudo et al., 2010; Zhelyaznik et al., 2003; Zhelyaznik and Mey, 2006; Nagashima et al., 2009; Reimer et al., 2009), and thus I had hypothesized that these receptors may too be upregulated in the CNS following a nerve-crush injury. My data show that expression of RXR, but not RAR, is altered in response to a nerve-crush injury. Interestingly, RXR expression is downregulated in the CNS by 24 hours after multiple nerve-crush injuries. This finding, while unexpected, may be explained by the time

course of these experiments. While substantial studies have shown that the retinoid receptors are upregulated in response to neural trauma (Agudo et al., 2010; Zhelyaznik et al., 2003; Zhelyaznik and Mey, 2006; Nagashima et al., 2009; Reimer et al., 2009) these previous studies typically investigated expression at least 3 days after injury. Thus, the expression level of the retinoid receptors over the first 24 hours after a nerve-crush injury requires further investigation. It is possible that some of the retinoid receptors are transiently downregulated following a nerve-crush injury, representing an immediate, early response to injury which may then be followed by a gradual upregulation, as is typically seen in other studies. Continued studies with *Lymnaea* examining extended time points beyond the first 24 hours will be required to validate this hypothesis.

Alternatively, it may be possible that a downregulation of the RXR is required to elicit regenerative responses. Work performed utilizing the newt has shown that the RXR is downregulated after injury (Carter, 2011). Given that the newt readily regenerates amputated limbs and thus is considered "regeneration-capable", this injury-induced downregulation of the RXR may be an essential factor in inducing regeneration. It would be expected that results obtained from a regeneration-capable animal may contradict studies utilizing animals such as rats (Zhelyaznik and Mey, 2006; Schrage et al., 2006)) which are not considered regeneration-capable. Moreover, it would appear that the RAR is critical in newt regeneration, as RAR antagonists can block the regeneration of the limbs (Carter, 2011) as well as RA-induced outgrowth of spinal cord explants (Dmetrichuk et al., 2005). Thus, in regeneration-capable animals, a downregulation of the RXR may be required to facilitate the effects of the RAR. Given that *Lymnaea* is thought to be regeneration-capable (Lukowiak et al., 2003; Lee and Syed,

2004; Syed et al., 1992), my data adds support to the notion that a downregulation of the RXR may be required, at least initially, for a regenerative response in regeneration-capable animals.

Interestingly, when comparing both the uncrushed and crushed CNS exposed to RA, I observed no alterations in either RAR or RXR expression when compared to EtOH controls. Previous work has shown that expression of both RAR in rat (Kamei et al., 1993; de et al., 1990) and RXR in mice (Bonet et al., 1997) can be modulated by RA exposure. Thus I had hypothesized that a morphological response induced by RA-exposure (increased outgrowth from RPeD1) may require an accompanying change in the expression of the retinoid receptors. It was expected that RAR or RXR expression would be increased in the RA-exposed CNS, which was not the case. Moreover, when studies were continued utilizing immunohistochemistry on whole CNS preparations, no difference was found in RXR expression at the crush site. Given that previous work has shown cytoplasmic retinoid receptor localization in response to injury (Zhelyaznik and Mey, 2006; Agudo et al., 2010), specifically at the site of neural damage, this result was unexpected. As with the previous section, expression in such studies was examined many days after injury, whereas in my experiments I examined expression at the 24 hour time point, and thus continued studies examining longer time points may provide insight into whether the RXR indeed localizes to the site of neural damage. Additionally, the present results can potentially be explained by three alternative hypotheses, discussed below.

First, and arguable most likely, there was an effect of EtOH alone (the vehicle) on the expression of the RXR. This result, while unexpected, is not without precedent, as Kumar *et al.*, (2010) have shown that EtOH exposure can indeed change the expression of the retinoid receptors in rat. Similar work has also shown that EtOH can compete with retinal for RALDH activity, and thus alter RA signaling during development (Kot-Leibovich and Fainsod, 2009). Thus, it would

appear that utilizing EtOH as a vehicle for RA may confound the ability to detect RA-specific effects. This suggestion is supported by my work showing that DMSO (another commonly used vehicle for RA) as well as EtOH impaired the crush-induced decrease in RXR expression. In these experiments, multi-crush preparations were expected to show a marked decrease in RXR expression after 24 hours (as seen in the absence of any vehicle); however, both EtOH and DMSO exposure appeared to prevent the decrease in RXR expression.

Secondly, Western analysis and immunohistochemistry procedures do not provide an indication of the endogenous activity of the RXR or RAR. While RXR expression remained relatively unchanged in the crushed CNS despite the application of RA or EtOH, it is possible that RA application alone may have increased the transcriptional activity of the RXR. Similarly, while I have shown no apparent change in the expression of the RAR (in both RA, EtOH and "no vehicle" conditions) this does not necessarily rule out the possibility that RA may elicit its effects through the increased transcriptional activation of this receptor. In other words, it is possible that either of the retinoid receptors, while unchanged in expression, may alter its transcriptional activity in response to RA application. Continued experiments utilizing a RARE driven reporter gene, as performed elsewhere (Saito et al., 2010), could verify these speculations. Thus, while RAR and RXR expression appear to be unaltered in response to RA exposure, an increased transcriptional activation of target genes in response to a nerve-crush injury cannot be fully ruled out at this time.

Thirdly, RA may be acting through a non-classical (ie: non-transcriptional) pathway independent of genomic RAR/RXR signaling. Our lab has previously shown novel effects of RA on growth cone turning. More specifically, RA can elicit positive growth cone turns from cultured neurons

which have had their cell bodies severed from the extending neurite (Dmetrichuk et al., 2006a). Moreover, it has been shown that the RXR is present in growth cones of regenerating processes of *Lymnaea* (Carter et al., 2010), suggesting a novel, non-genomic role during regeneration. Thus, RA could elicit some effects through a currently unknown pathway, either via a non-genomic action of a retinoid receptor or via an alternative signaling pathway independent of either receptor. If indeed this is the case, RA would not appear to require the classical transcriptional activation or changes in receptor expression levels. Further work would be needed to address these speculations.

Lastly, given their involvement in the retinoid signaling pathway, specifically their upregulation in response to neural injury (Zhelyaznik et al., 2003), it would have been beneficial to investigate the regulation of both RALDH and CYP26. Unfortunately, this was not possible due to the lack of commercially available antibodies which are effective in *Lymnaea*. Furthermore, while the full length mRNA sequence is known for RALDH in *Lymnaea*, the full length sequence for CYP26 is currently unknown. At this time, these studies were thus limited to investigating the retinoid receptors, for which their full length sequences are known, and for which custom made antibodies are available for use in *Lymnaea*.

In summary, RA exposure has been shown to increase the total length of RPeD1 neurites in a nerve crush-dependent manner. Other similar work with vertebrate dopaminergic neurons has shown that these neurons are responsive to RA or RA analogues. In such studies, application of RA (Ulusoy et al., 2011) or RA analogues (Katsuki et al., 2009) was found to prevent dopaminergic neuron loss and preserve dopaminergic function, respectively. These previous studies, and many others, however, have investigated arguably more large scale outcomes within

an animal as opposed to a detailed analysis of the specific interactions RA has within cells. My current work offers the ability to characterize the effects RA has on single dopaminergic neurons. RA signaling is thought to play a role in a number of neurological diseases that affect dopaminergic neurons such as Parkinson's disease (Krezel et al., 1998), schizophrenia (Krezel et al., 1998) and depression (Bremner and McCaffery, 2008). These findings support the use of this animal model and the nerve-crush injury paradigm in the continued study into the underlying interactions between RA and injured dopaminergic neurons. These studies also provide the first *in situ* evidence that RA can affect neurite outgrowth in an intact invertebrate CNS.

Chapter 3

The characterization of a novel RA-induced electrophysiological change in identified dopaminergic and peptidergic neurons.

3.01 – Abstract

The electrical activity of neurons is well known to play a critical role in neuronal development, guiding the proper formation of neural circuits and is thought to play a role during neuronal repair in adult nervous tissue. For example, the extension of neurites and motility of growth cones has been shown to be modulated by changes in the electrical activity of neurons. RA is known to elicit regenerative responses, namely induction, enhancement and direction of neurite outgrowth . The previous chapter has shown that RA can also elicit a change in neurite morphology *in situ,* promoting the extension or preservation of fine processes of injured RPeD1 neurons (Chapter 2). Currently, the mechanisms underlying this RA-induced, crush-dependent change in neurite morphology remains unclear. The data suggest that it may not require significant changes in the expression of the retinoid receptors or the localization of the RXR to the crush site, suggesting that RA may operate outside of the classical retinoid signalling pathway. Given that RA and the electrical activity of neurons can elicit similar morphological responses, such as inducing neurite outgrowth, the next aim of this thesis was to determine whether exposure to RA could alter the firing properties of nerve-crushed RPeD1 neurons. The data show, for the first time in an invertebrate species, that the application of RA can rapidly elicit dramatic changes in the firing properties of neurons. First, application of RA causes the presence of atypical firing behavior, whereby RA-exposed neurons exhibit rhythmic bursting. Secondly, RA exposure alters the shape of action potentials, causing increases in the half-amplitude duration and decay time. Lastly, RA causes a cell silencing effect whereby neuronal activity is halted within an hour after RA-exposure. The effects of RA are shown to be maintained across multiple cell types and are both dose-dependent and isomer-dependent. The effects of RA on cell firing suggests that some of its effects on outgrowth may be mediated by changes in the firing properties of injured neurons.

3.02 - Introduction

The previous chapter has shown that RA elicits a change in neurite morphology in injured dopaminergic neurons. More specifically, a nerve-crush injury paradigm was designed which causes the degeneration of fine neurite processes of RPeD1. This neurite degeneration is not observed in the presence of RA, suggesting that RA has the capacity to either induce new outgrowth or to preserve existing neurites. The mechanisms by which RA elicits this response remain unclear. Despite a crush-dependent alteration of RXR expression in the CNS, there does not appear to be any recruitment of the RXR to the crush site. Even though these studies did not rule out a transcriptional role of RA, it is possible that RA may be exerting its effects through many other non-transcriptional pathways. According to the literature, one aspect that has not yet been studied is the possibility that RA may mediate its morphological effects via changes in the electrical properties of neurons.

The electrical activity of neurons is well known to play a critical role in neuronal development, more specifically guiding extending neurites to their proper synaptic targets (Crair, 1999). For example, it has been well established that both spontaneous as well as experience-based (ie: induced) neural activity are essential for the proper formation of synaptic connections in developing mammals (reviewed extensively by Katz and Shatz (1996)). Impairment of firing activity typically results in a lack of formation of cortical networks in, for example, those required for visual processing (Katz and Shatz, 1996). Thus, despite the presence of other developmental factors, the firing activity of neurons would appear to be essential for the proper guidance and establishment of synaptic connections during development. If regeneration is thought to recapitulate developmental processes it would be reasonable then to assume that the electrical activity of neurons may play a critical role during regeneration as well. No work to

date has investigated whether exposure to RA can elicit changes in the firing pattern of regenerating neurons. The mechanisms underlying RA's regenerative effects on either vertebrate or invertebrate neurons are largely unknown; however, one possibility is that RA may alter the firing properties of neurons. Thus the downstream effects of RA may include alterations in how neurons fire action potentials, which in turn may modulate neurite outgrowth and growth cone guidance during regeneration.

The notion that the electrical activity of neurons can modulate a regenerative response to injury is supported by various findings in the literature. For example, action potentials from injured neurons have been shown to have a reduced afterhyperpolarization in the lamprey (McClellan et al., 2008). Similar work in mice has demonstrated that extension of neurites (Lautermilch and Spitzer, 2000) as well as growth cone motility (Ibarretxe et al., 2007a) can be modulated by changes in the electrical activity of neurons. Moreover, work in *Helisoma* has shown that application of growth-inhibiting and growth-enabling substances (serotonin and acetylcholine, respectively) rapidly alters action potential firing (McCobb et al., 1988). These studies have shown that increasing the firing rate of neurons can result in a "stalling" of growth cone extension, whereas reducing action potential firing typically leads to increased rates of outgrowth. This stimulation-mediated growth cone stalling is often reversible, showing that growth cone motility can be actively regulated by the firing state of neurons. Additionally, work utilizing *Xenopus* spinal neurons has shown that the firing state of a neuron can have a dramatic impact on the sensitivity of growth cones to guidance cues, as well as modulate the type of response (ie: attractive/repulsive growth cone turn) elicited by a guidance molecule (Ming et al., 2001). These studies highlight the impact of electrical activity on regenerating neurons, because the same guidance molecule might elicit very different responses, depending on the firing state

of the neuron. Taken together, these studies show that the firing activity of neurons appears to play a critical role in the extension and guidance of regenerating neuronal processes.

Interestingly, our lab has previously shown that RA can induce and enhance neurite outgrowth as well as direct growth cone guidance in cell culture (Dmetrichuk et al., 2006a; Dmetrichuk et al., 2008). These are all processes which are also modulated by changes in the electrical firing of the cell. While RA is known to play a role in regeneration, few studies have investigated its ability to alter the electrophysiological properties of cells, and many of these have not examined neurons. For example, exposure to RA has been shown to cause changes in the ion channel densities of cultured human neuroblastoma (Tonini et al., 1999) and SH-SY5Y (Arcangeli et al., 1998) cells; however, these studies are confounded by the fact that these cells were actively differentiating during RA exposure. Similarly, work utilizing human embryonic kidney cells has shown that application of RA can lead to a rapid hyperpolarization of the resting membrane potential (Xiao et al., 1998). Work performed in the striped hybrid bass has shown that RA application can lead to a decrease in electrical coupling in retinal cells (Zhang and McMahon, 2000). Unfortunately this work did not investigate whether the change in coupling resulted in changes in the firing pattern of these neurons, and whether this response was altered during neuronal injury. Taken together, the above studies suggest that RA has the ability to alter the electrophysiological properties of some cells. Currently however, no studies have examined whether application of RA can lead to electrophysiological changes in regenerating adult neurons.

Overall, very little work exists to date that has studied whether RA can elicit a change in the electrical properties of neurons. Previous work using *Lymnaea* has shown that cultured neurons exposed to RA remain electrically functional for longer durations than control neurons cultured

in the absence of RA (Dmetrichuk et al., 2006a). Specifically, Dmetrichuk et al. (2006) showed that visceral F (VF) neurons maintained significantly more negative resting membrane potentials in the presence of RA. Additionally, RA-exposed VF neurons were more likely to fire action potentials (either spontaneously or when evoked via depolarizing current injection) than control neurons. These data show that in *Lymnaea*, RA appears to preserve the electrical properties of identified neurons over many days in culture. However, whether RA can elicit acute changes in firing properties that may underlie regenerative responses, has not previously been studied.

The main aim of this chapter, therefore, was to determine whether acute RA exposure could indeed induce any changes in the firing properties of adult neurons, and if so, to determine if these responses are injury-dependent. To this end, the first aim of this chapter was to determine whether RA can alter the firing properties of injured adult neurons within the CNS utilizing the *in situ* nerve-crush injury preparation used in Chapter 2, as this injury model results in the degeneration of RPeD1 neurons. In an effort to determine whether any RA-induced changes in firing properties are cell type specific, the identified VF neurons which, in culture, have been shown to be responsive to RA (Dmetrichuk et al., 2006b), were also examined. Second, in an effort to characterize any RA-induced changes in firing properties, cell culture was utilized to investigate RA's effects on both RPeD1 and VF neurons.

3.03 - Materials and Methods

CNS isolation and Nerve-crush. All procedures and equipment used were identical to those described previously in Chapter 2 unless otherwise stated. Crushed CNS preparations consisted of two crushes. The first crush was performed as in Chapter 2, between the parietal and pleural ganglia, to crush RPeD1's main axon. The second crush was induced between the left parietal and visceral ganglia, to crush the axons of VF neurons which project into the intestinal, left parietal and median inferior pedal nerves (Syed and Winlow, 1991). Crushes were performed immediately after the CNS was removed from the animal. As in the previous chapter, the uncrushed CNS condition was used to control for the removal of the CNS from the animal. Following CNS removal and nerve crushes, intracellular recordings were made from both RPeD1 and VF neurons for 10 minutes prior to and up to one hour after exposure to 10μM RA or EtOH (vehicle) control. This concentration was increased from 1μM RA used in the previous chapter in an effort to amplify any subtle electrical changes that may result from RA exposure. CNS preparations, if left for 24 hours, were incubated, unpinned, in defined medium (DM). During electrophysiological recordings, CNS preparations were bathed in saline.

Cell Culture. Laboratory reared *Lymnaea stagnalis* were housed in artificial pond water and fed lettuce. Cell culture techniques were performed as described previously (Dmetrichuk et al., 2006a). Briefly, animals were anesthetized, and the central ring ganglia were removed and bathed in antibiotic saline containing 225μg/ml gentamycin. Ganglia were then trypsinized (2mg/ml DM) for 19 minutes, pinned out in high osmolarity defined medium (L-15 medium) and the outer sheath of the ganglia was removed. The inner sheath encapsulating the ganglia was then removed and the somata of identified Visceral F (VF) or Right Pedal Dorsal 1 (RPeD1) neurons were removed via suction with a fire polished pipette. Neurons were plated on poly-L-lysine

coated culture dishes containing 3ml of DM unless stated otherwise, and then incubated at 21°C overnight.

Electrophysiology. Glass electrodes ranging from 20 to 40MΩ in resistance were pulled using a Kopf pipette puller (Model 730, David Kopf Instruments, California, USA) and backfilled with a saturated potassium sulphate solution. Recordings were made using an intracellular recording amplifier (Neuro Data IR283A, Cygnus Technology Inc., Pennsylvania, USA) and a Powerlab 4sp data acquisition system running Chart v4.2 (AD Instruments, Colorado, USA). *In vitro* recordings were made from individual neurons after approximately 24 hours in culture. Cell activity was recorded in DM for approximately 10 minutes prior to the addition of either retinoid or EtOH (vehicle control). Agents were administered by gently pipetting 300µl of a working stock solution (at a concentration ten times higher than the final bath concentration) along the perimeter of the culture dish, which contained 2.7ml of media. After addition, the pipette was gently aspirated and pipetted 8-10 times to equally distribute the working stock solution throughout the culture dish. The resting membrane potential and cell activity were then recorded for a further 60 minutes. During this time, the membrane potential was not altered, except briefly at specific time points following RA (or vehicle) addition (2.5, 15, 35, 50 and 60 minutes after addition). At these specific times, the membrane potential was manipulated (using current injection) to reach firing threshold and allow the cell to fire 5-10 action potentials at a frequency of ~ 1Hz or less. Following this, the neuron was briefly depolarized (typically by 50-100pA in two separate steps) to induce a brief, rapid firing response for approximately 20 seconds. Current injection was then stopped and the membrane potential was allowed to return to its resting value until the next time point for manipulation. The input resistance of cultured neurons was measured by five stepwise injections of hyperpolarizing current, ranging from 50pA to 250pA, in

50pA steps. Input resistance measurements were taken immediately prior to and one hour after addition of RA or EtOH. Frequency-dependent impulse broadening was performed using spike trains of similar frequency between groups. The mean spike train frequency was not significantly different between groups in any comparison.

Chemicals. All chemicals were purchased from Sigma-Aldrich unless otherwise stated. *All-trans* and 9*cis* retinoic acid working stocks (10mM) were made fresh daily.

Spike Waveform Analysis. Firing activity was analyzed qualitatively before and after RA exposure, and single action potentials were quantitatively analyzed at various time points after addition of RA. Chart v4.2 (AD Instruments, Colorado, USA) software was used to analyze components of the spike waveform. At each time point, three individual action potentials were analyzed, and the data were averaged as representative examples for each firing characteristic. Rise time is defined as the time from 10% to 80% of the spike amplitude on the leading edge of the action potential. Decay time is defined as the time from 10% to 90% of the falling phase of the action potential. The half-amplitude duration is the time between the midpoint of the leading and falling edges of the action potential. Frequency dependent broadening was analyzed by comparing the half-amplitude duration of the first and third action potentials in a train of action potentials. The percent broadening was calculated as the percent change in half-amplitude duration of the third action potential compared to the first action potential. Since few cells fired trains of action potentials by 35 minutes after RA exposure, this analysis was performed at the 15 minute time point only.

Statistical Analysis. Analysis of data was performed with SigmaStat v3.5 software (SigmaStat Software Inc., Virginia, USA). For experiments studying changes in firing properties in crushed vs. uncrushed CNSs differences between RA and EtOH-exposed CNS preparations were determined by using a t-test within each crush condition. For experiments conducted in cultured neurons, differences between groups at specific time points were determined using a one way ANOVA on data normalized to pre-exposure values (set at 0% for graphical data and 100% for tabulated data, unless stated otherwise). If significant differences were found ($p < 0.05$) a Tukey-Kramer *post hoc* test was performed. Any data set that did not meet the assumptions of the ANOVA were either log or reciprocally transformed prior to further analysis. In the event that data did not meet normality conditions, a one way Kruskal-Wallis ANOVA on ranks was performed followed by a Dunn's *post hoc* test. The presence or absence of atypical impulse activity for each cell was analyzed using Fisher's Exact tests, which were then Bonferroni-Holm corrected. All values are expressed as mean ± standard error of the mean (S.E.M.), unless otherwise stated.

3.04 – Results

I. Acute RA exposure causes electrophysiological changes in VF, but not RPeD1 neurons

In the following series of experiments, I investigated whether RA could induce electrical changes in RPeD1 neurons (which were used in the previous chapter) and VF neurons, which have been shown to be responsive to RA in cell culture (Dmetrichuk et al., 2006a). As nerve injury can affect both neuronal firing patterns and action potential shape (referred to hereafter as spike waveform) (McClellan et al., 2008), both of these parameters were measured for any responses to RA. Firing activity was analyzed qualitatively before and after RA exposure, and single action potentials were quantitatively analyzed at various time points after addition of RA. The action potential parameters that were measured include the peak to peak amplitude, rise time, decay time and half amplitude duration. Furthermore, to determine whether RA elicited changes in intrinsic membrane properties, the input resistance and resting membrane potential (RMP) of neurons were measured prior to and one hour after application of RA or EtOH (vehicle control). In each cell, changes in the AP parameters measured in response to RA were compared to values measured before application of RA (considered as the "baseline" values).

Neither crushed nor uncrushed RPeD1 neurons showed any response to 10μM RA (crushed: n = 10; uncrushed: n = 11) or 0.1% EtOH (crushed: n = 10; uncrushed: n = 8) in terms of firing pattern (Figure 3.1). Likewise, as shown in Figure 3.1, neither crushed nor uncrushed VF neurons showed any obvious response to RA (crushed: n = 17; uncrushed: n = 11) or EtOH (crushed: n = 13; uncrushed: n = 11). When analyzing both uncrushed and crushed RPeD1 neurons, no significant differences were found for any spike waveform parameters at the 15, 35 or 50 minute time points after exposure to 10μM atRA or EtOH control (t-tests performed for each parameter within each crush condition; 35 minute data presented in Appendix 1.02). Results

obtained for the half-amplitude duration and decay time 15 minutes after exposure are shown in Figure 3.2. Similar results were obtained for VF neurons at the same time points (Figure 3.2; t-tests performed for each parameter within each crush condition). Additionally, no change in the resting membrane potential of RPeD1 or VF neurons exposed to RA was found when compared to EtOH control, regardless of the crush condition (Appendix 1.03). Taken together these data suggest that, with or without axonal injury, RA exposure did not alter the spike waveform of either RPeD1 or VF neurons within the first 50 minutes of exposure. However, VF neurons from uncrushed as well as crushed CNS preparations showed a significant increase in half-amplitude duration when exposed to RA for 60 minutes; there was no such change in RPeD1 neurons in either crush condition. Additionally, the decay time of action potentials from VF neurons exposed to RA for 60 minutes was significantly increased in the nerve-crushed CNS. This difference was not seen in VF neurons from uncrushed preparations; however, a modest trend was observed in which RA-exposed neurons displayed slightly increased decay times when compared to controls. No change was observed in the decay time of RPeD1 neurons in either crush condition (Figure 3.3).

The above data show that (at the level of the soma) VF, but not RPeD1 neurons show subtle, yet statistically significant increases in the half-amplitude duration and decay time of action potentials in response to acute RA exposure. I have previously shown that RA increases the length of fine processes of RPeD1 in the pedal ganglion by 24 hours after a nerve-crush injury. Since the morphological response to RA in these previous experiments was examined 24 hours after injury, it is unclear when RA initiated such effects. Thus, I next sought to determine whether extending the latency between nerve-crush injury and RA exposure would alter either

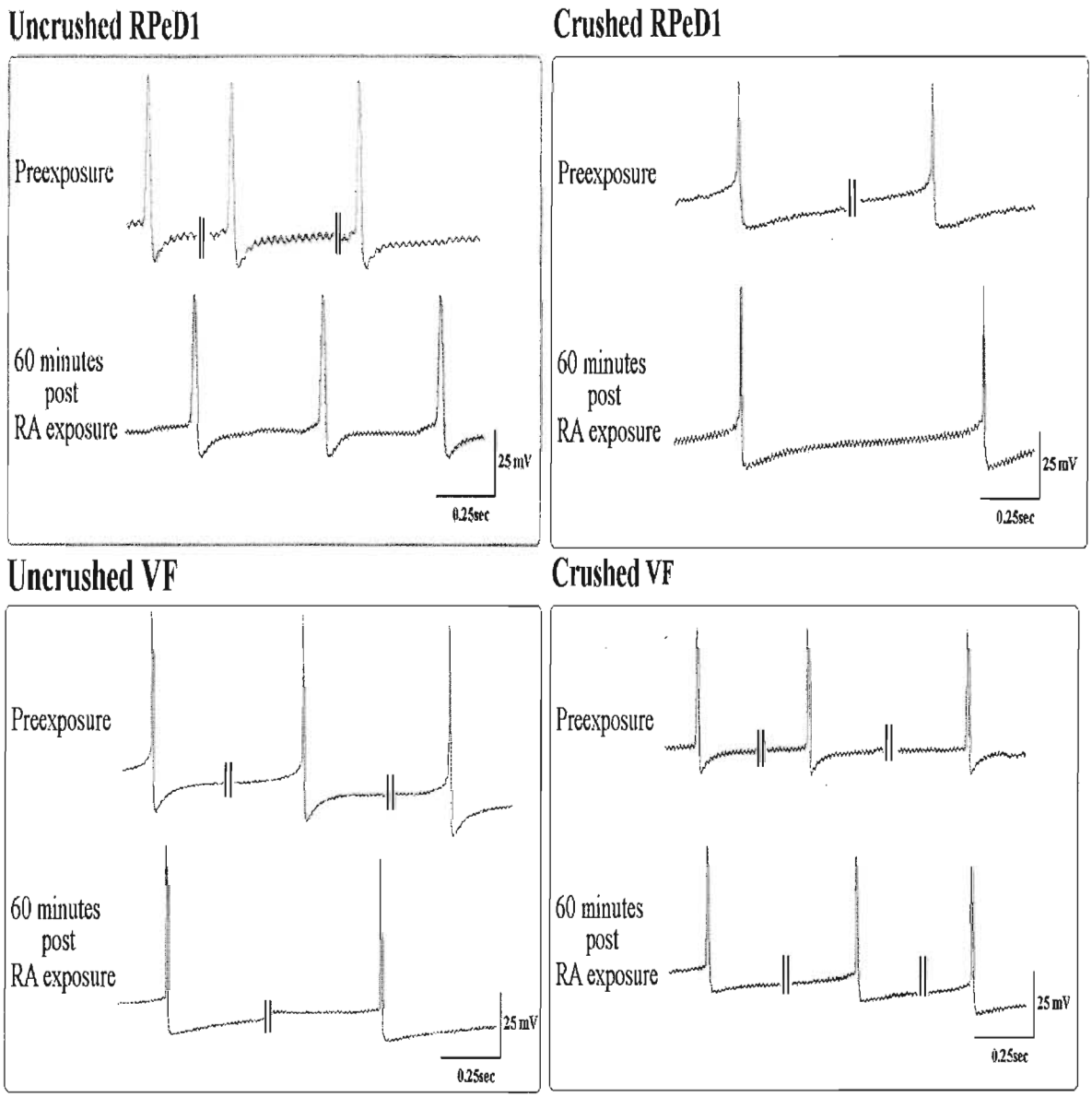


Figure 3.1. RA-exposure does not produce dramatic changes in the shape of action potentials from identified RPeD1 or VF neurons in the intact CNS. Representative recordings from uncrushed (left) and crushed (right) CNS preparations of RPeD1 (upper) and VF (lower) neurons prior to and one hour after exposure to 10μM atRA. In all cases firing properties appear to remain unchanged. || denotes a break in the recording.

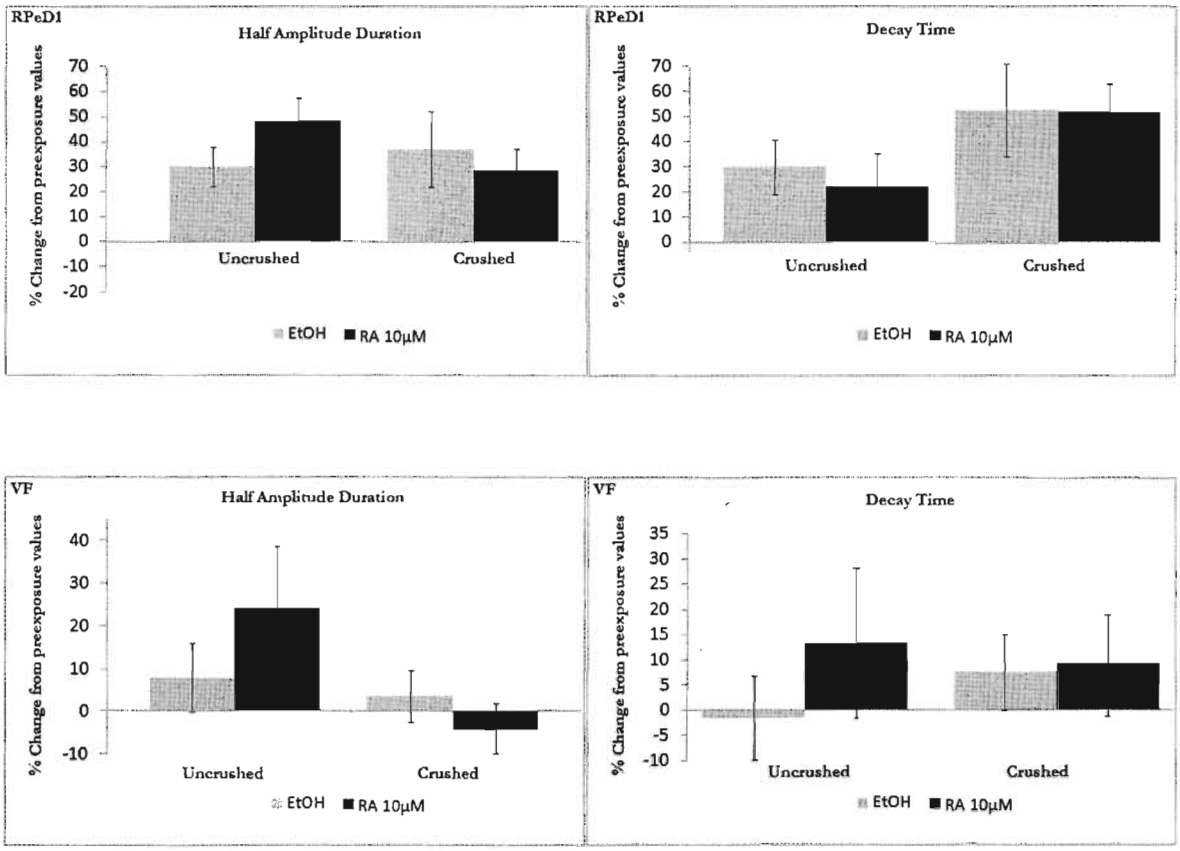


Figure 3.2. Spike waveform is unaffected by RA 15 minutes after exposure. The half amplitude duration (left) and decay time (right) of RPeD1 (upper) and VF (lower) neurons in uncrushed and crushed CNS. All values are calculated as a percent change from pre-exposure values (set at 0%) obtained 10 minutes prior to the addition of RA or EtOH. In all cases, no significant difference was found. Replicates for RA and EtOH-exposed RPeD1 neurons in uncrushed CNS were 10 in both cases. Replicates for RA and EtOH-exposed RPeD1 from crushed CNS were 11 and 8, respectively. Replicates for both RA and EtOH-exposed VF neurons in uncrushed and crushed CNS were 13 and 17, respectively.

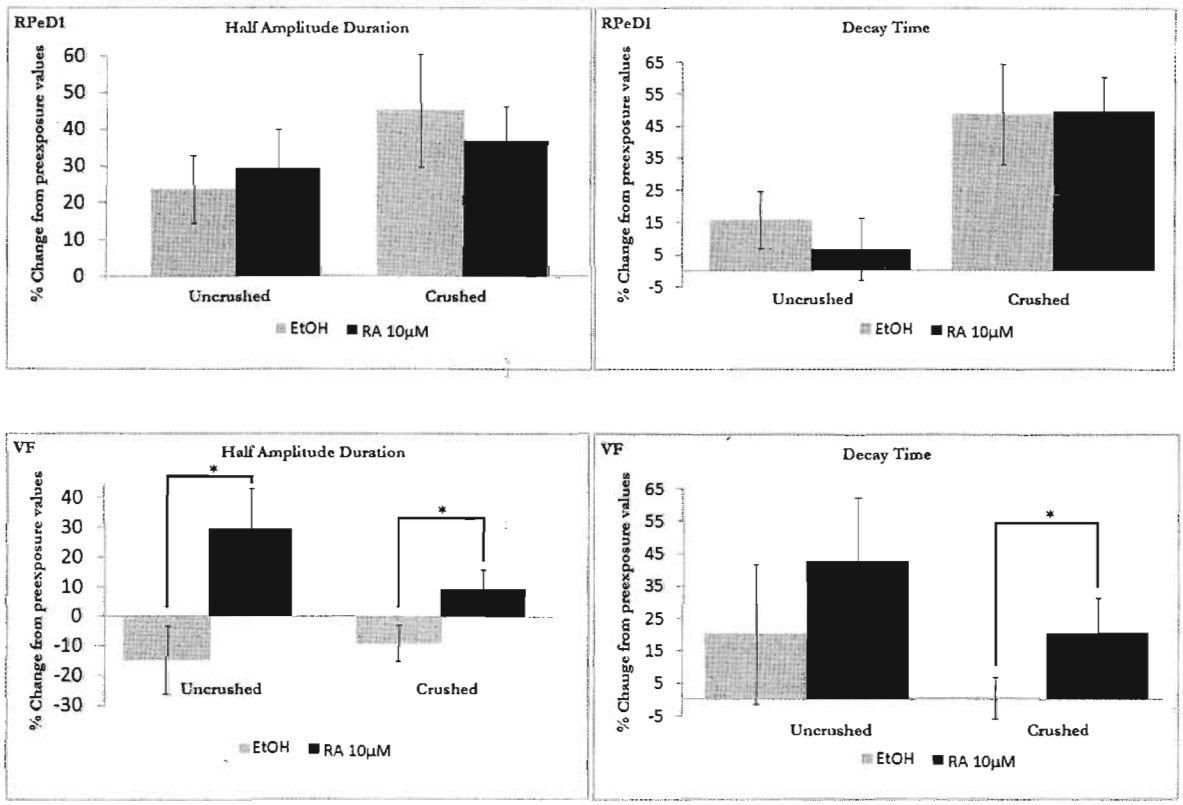


Figure 3.3. RA-exposure increases the half-amplitude duration and decay time of VF neurons 60 minutes after exposure. No significant difference was found in either the half-amplitude duration (upper left) or decay time (upper right) of RPeD1 action potentials in uncrushed or crushed CNS. The half-amplitude duration (lower left) of RA-exposed VF neurons was significantly increased 60 minutes after application in both uncrushed and crushed CNS. VF neurons in crushed CNS alone showed a significant increase in decay time (lower right). All values are calculated as a percent change from pre-exposure values (set at 0%) obtained 10 minutes prior to the addition of RA or EtOH. Number of replicates per condition is the same as in Figure 3.1. (*=$p<0.05$).

RPeD1 or VF responsiveness to acute RA application. In these experiments, the CNS was crushed and then incubated in DM for 24 hours prior to the start of electrophysiological recordings. Following this incubation, RPeD1 and VF neurons were impaled and exposed to 10μM atRA or EtOH as performed previously. In addition, I performed an identical series of experiments on uncrushed CNS incubated for 24 hours in DM in parallel, in order to determine whether any RA-induced changes were crush-dependent.

As before, neither crushed nor uncrushed RPeD1 or VF neurons showed any changes in their firing pattern in response to RA or EtOH (Figure 3.4; n = 8 in all conditions). When analyzing the spike waveform of RPeD1 neurons exposed to RA, no significant differences were observed in either uncrushed or crushed CNS when compared to controls. Examples of the half-amplitude duration and decay time from RPeD1 neurons in uncrushed and crushed CNS are shown for 15 and 60 minutes time points in Figures 3.5 and 3.6, respectively. In sharp contrast to the above results, the half-amplitude duration and decay time of crushed VF neurons were found to be significantly increased compared to EtOH controls as early as 15 minutes after RA exposure, but no such effects were yet apparent in uncrushed VF neurons at this time point (Figure 3.4). In all cases, no differences were found for the peak to peak amplitude or rise time of action potentials at any time point (Appendix 1.04). When analyzing spike waveform 35 and 50 minutes after RA exposure, it was found that VF neurons from both crushed and uncrushed CNS showed significantly increased half-amplitude durations and fall times when compared to EtOH controls (Appendix 1.04). While similar results were also obtained for the 60 minute time point, only the RA-exposed VF neurons from uncrushed CNS showed statistically significant differences (Figure 3.5). This indicates that RA initiated changes in action potential waveform more rapidly

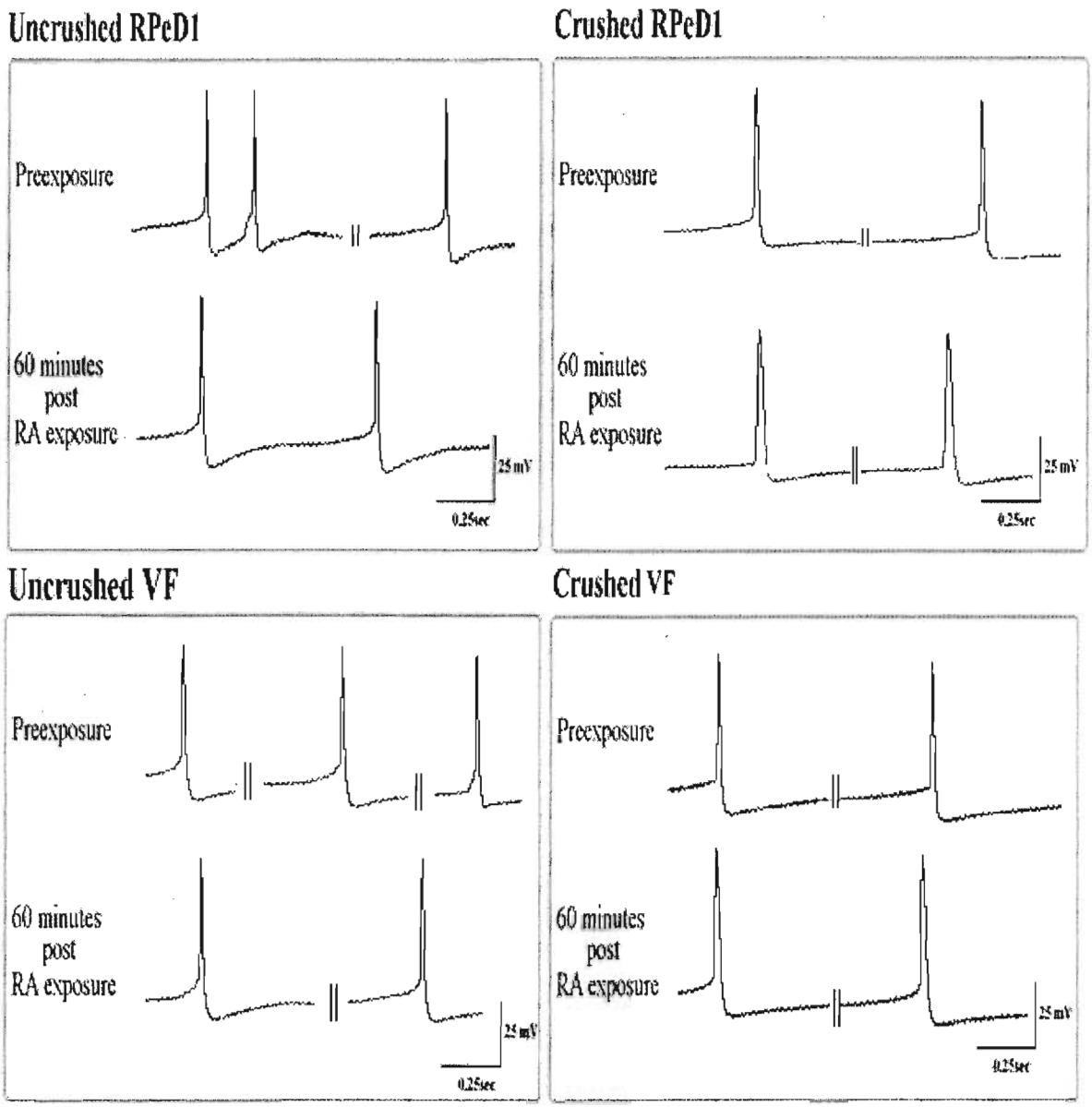


Figure 3.4. RA-exposure does not produce dramatic changes in the shape of action potentials from identified RPeD1 or VF neurons in the intact CNS 24 hours after CNS isolation and application of a nerve-crush injury. Representative recordings from uncrushed (left) and crushed (right) CNS preparations of RPeD1 (upper) and VF (lower) neurons prior to and one hour after exposure to 10μM atRA. In all cases firing properties appear to remain unchanged. || denotes a break in the recording.

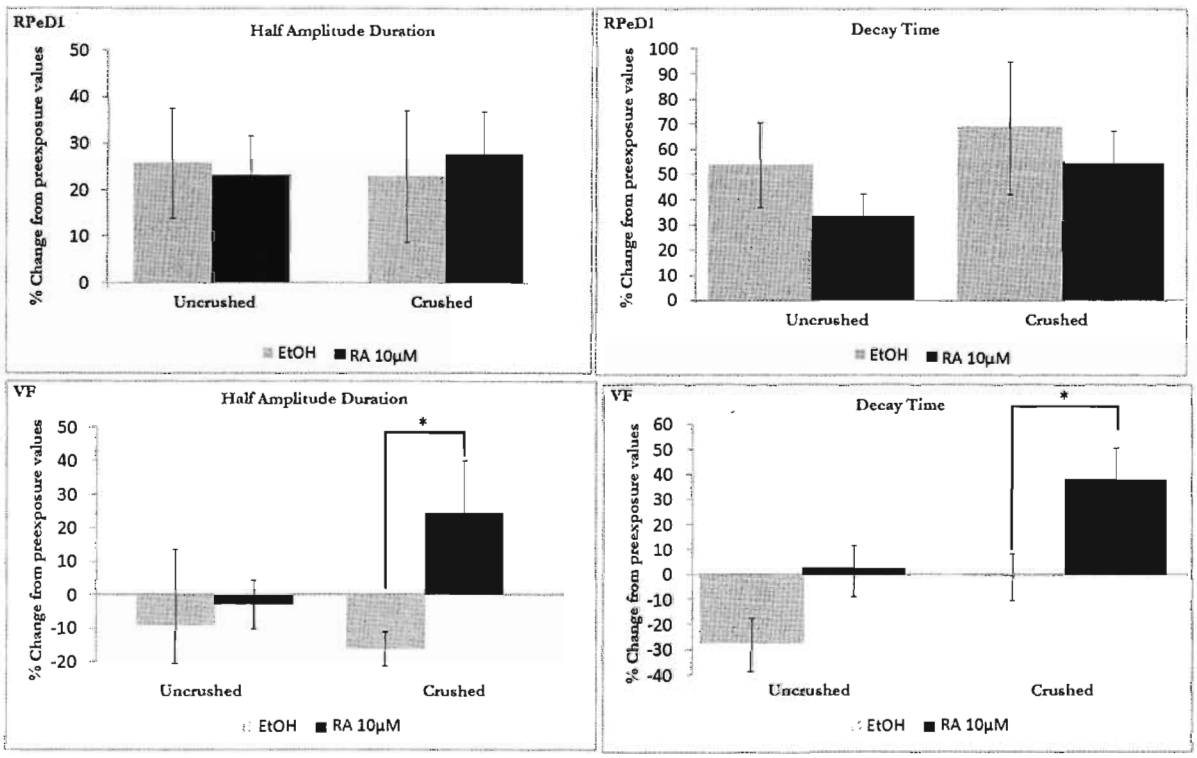


Figure 3.5. 15 minute RA exposure increases the half-amplitude duration and decay time of crushed RPeD1 and VF neurons 24 hours after CNS isolation. The half amplitude duration (left) and decay time (right) of RA-exposed RPeD1 (upper) and VF (lower) neurons 15 minutes after application, expressed as a percent change from pre-exposure values (set at 0%). The half amplitude duration and decay time of RA-exposed VF neurons in the crushed CNS are significantly increased 15 minutes after retinoid exposure. RA had no such effects on RPeD1 or on either neuron in uncrushed CNS isolated for 24 hours. n=8 for all conditions. (*=$p<0.05$)

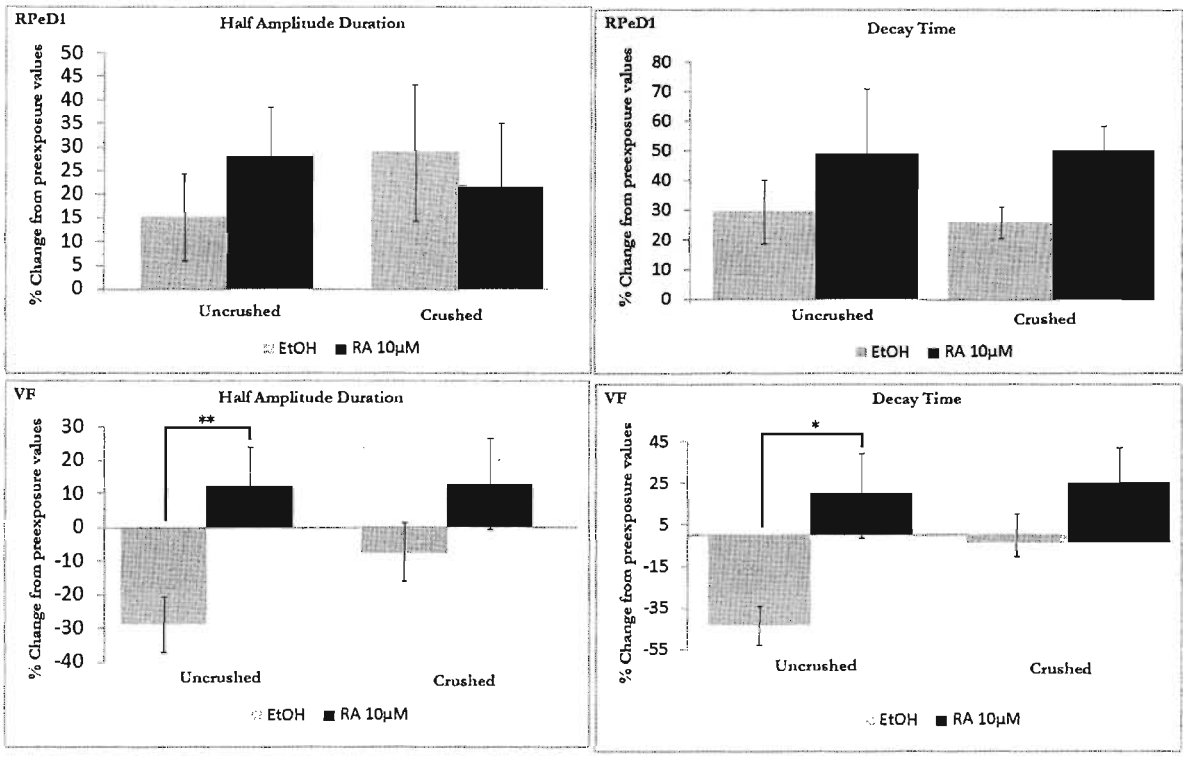


Figure 3.6. Effects of 60 minute RA exposure on half-amplitude duration and decay time of RPeD1 and VF neurons 24 hours after CNS isolation with or without nerve-crush. No significant differences were found between RA and EtOH-exposed RPeD1 neurons (upper). The half amplitude duration (left) and decay time (right) of RA-exposed VF neurons (lower) from uncrushed CNS are significantly increased 60 minutes after retinoid exposure. Data are expressed as a percent change from pre-exposure values (set at 0%) n=8 for all conditions. (*=$p<0.05$, **=$p<0.01$)

in CNS preparations 24 hours after a nerve-crush injury. This strongly suggests that exposure to a nerve-crush injury increases the responsiveness of injured VF neurons to RA.

II. Acute RA exposure induces firing pattern changes in cell culture.

Results obtained with isolated CNS suggest that the response to RA may be cell type specific, as VF neurons (but not RPeD1) showed spike waveform changes following addition of RA. Additionally these data suggested that the response to RA was enhanced and/or occurred sooner if the CNS had been injured 24 hours earlier, compared to those tested one hour after nerve-crush injury. Aside from an accelerated time course, however, the nerve-crush injury did not alter the RA-induced changes, as increases in the half-amplitude duration and decay time were seen in both uncrushed and crushed CNS. Since the intact CNS possesses numerous signaling molecules, chemical and electrical interactions from synaptic contacts and many other modulatory factors, I next sought to extend these studies utilizing cell culture, where identified neurons could be placed in an environment devoid of such potentially confounding interactions and directly exposed to RA.

i. RA produces action potential widening in both VF and RPeD1 neurons.

As a first step in determining whether RA can elicit firing changes in neurons in culture, I sought to determine if acute exposure to RA altered the firing properties of identified neurons within the first hour of exposure. In these experiments VF and RPeD1 neurons were plated onto culture dishes and allowed to recover for 24 hours, after which time they were impaled for

electrophysiological recordings for 10 minutes prior to and up to one hour after the addition of RA or EtOH vehicle. As shown in Figure 3.7, application of 10μM RA caused changes in action potential duration in both VF (n=22) and RPeD1 (n=9) neurons. In both cases the onset of changes in action potentials was seen by 15 minutes after application of RA, the same time required for RA to increase impulse duration in the intact CNS, 24 hours after nerve-crush injury (Figure 3.5). In cell culture, however, RA application elicited changes in the action potential shape of both VF and RPeD1 neurons, whereas only VF neurons showed these changes in the intact CNS. All neurons exposed to RA appeared to have increased action potential duration as seen by the widening of individual action potentials (classified here as action potential widening) when compared to action potentials of the same cell prior to addition of RA (baseline). This effect was quantified and is described more fully in the following section. Additionally, a reduction in the magnitude of the afterhyperpolarization of the action potential was observed. Taken together, these data demonstrate that acute exposure to RA caused changes in the shape of action potentials in multiple neuronal cell types as early as 15 minutes after exposure.

ii. RA produces atypical impulse activity in both VF and RPeD1 neurons.

In addition to changes in action potential shape, RA-exposed neurons displayed a number of atypical firing patterns not previously observed in the intact CNS with RA exposure (Figure 3.8). Such atypical firing patterns included spike doublets, triplets and extended plateau potentials (cumulatively classified here as "atypical impulse activity"). Approximately 57% (16/28) of VF and 44% (4/9) of RPeD1 neurons exposed to RA displayed these atypical firing patterns, whereas only 4% (1/25) of VF and none (0/7) of the RPeD1 control cells (exposed to the vehicle, EtOH) displayed atypical firing patterns over the duration of the recording.

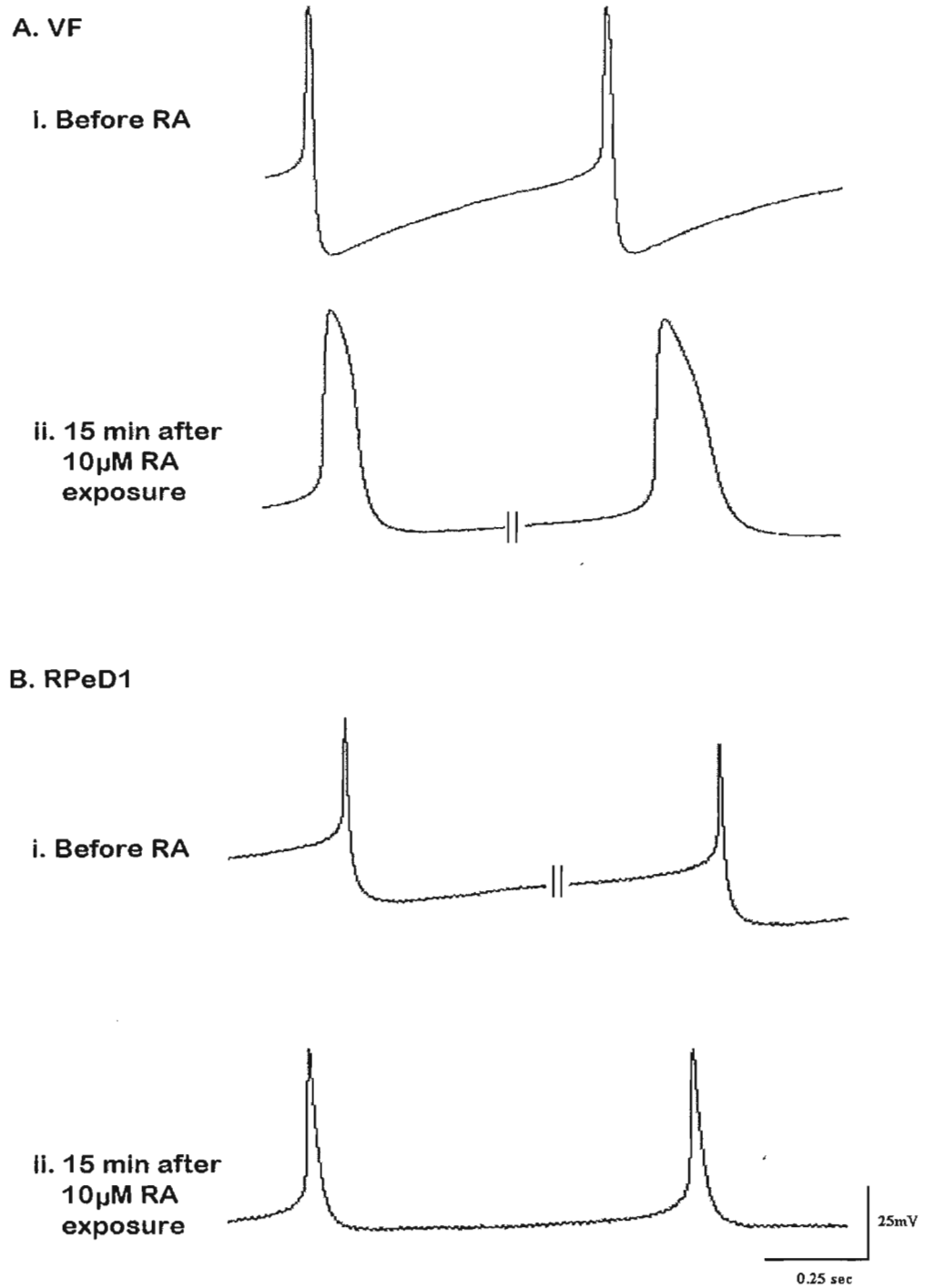


Figure 3.7. Exposure to RA causes action potential widening and reduced afterhyperpolarization within 15 minutes of exposure in cell culture. Representative recordings from VF (A) and RPeD1 (B) neurons acutely exposed to 10μM atRA. Approximately 15 minutes after exposure to RA (ii), neurons showed action potential widening and a reduction in afterhyperpolarization when compared to the same neuron prior to RA exposure (i). || denotes a break in the recording.

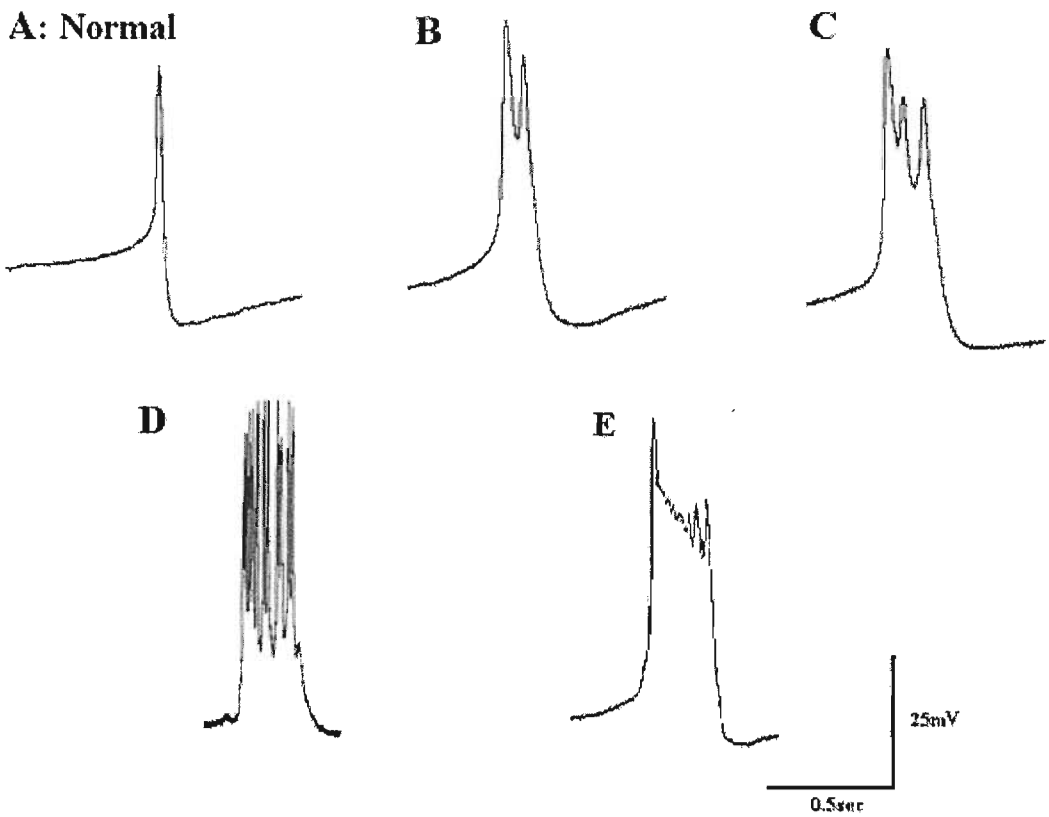


Figure 3.8. Representative examples of atypical firing patterns seen in RA-exposed VF cells in culture. A: normal action potential prior to the addition of 10μM atRA, B: spike doublet, C: spike triplet, D: bursting and E: extended plateau potential. B - E were observed in RA-exposed neurons.

iii. RA produces cell silencing in both VF and RPeD1 neurons.

While the shape of single action potentials and the firing behavior of neurons were altered within 15 minutes of exposure to RA, approximately 73% of VF neurons and 67% of RPeD1 neurons exposed to 10μM RA were unable to fire spontaneous or induced action potentials by 35 minutes after RA exposure. Furthermore, after one hour of exposure to RA nearly all VF (21/22) and RPeD1 (8/9) neurons were unable to fire action potentials either spontaneously or in response to depolarizing current injection. These data are in stark contrast to recordings from neurons in the intact CNS, where all RPeD1 neurons (10/10) and 91% of VF neurons (10/11) were able to fire induced or spontaneous action potentials 60 minutes after RA exposure. In cell culture, both VF and RPeD1 cells exposed to EtOH (vehicle control) continued to fire action potentials for the full duration of the recordings (VF: n= 22, RPeD1: n=7). These data demonstrate that acute exposure to RA caused dramatic changes in the shape of action potentials and a dramatic change in firing behavior, while simultaneously causing neurons to become silent within the first hour of exposure.

The RA-induced cell silencing could be the result of changes to the resting membrane potential (RMP). Specifically, if RA exposure caused a gradual depolarization of the RMP to levels where sodium channels inactivate, RA-exposed neurons would be unable to fire. To determine if this was the case, I next examined the RMP prior to and one hour after exposure to RA or vehicle (EtOH). RPeD1 neurons exposed to either RA or EtOH (control) were found to have virtually identical RMPs (RA: -65 ± 5 mV; EtOH: -65 ± 5 mV; n=6 for both groups) prior to their respective treatment; however, after one hour of exposure to RA, the RMP hyperpolarized to -70 ± 3 mV whereas in EtOH controls, the RMP depolarized to -56 ± 4 mV (Figure 3.9, A). Likewise, VF neurons in the RA or EtOH groups were found to have virtually identical RMPs

prior to exposure (RA: -66 ± 5 mV, n=23; EtOH: -64 ± 4 mV, n=19), which became hyperpolarized in RA-exposed neurons (-74 ± 3 mV) and depolarized in EtOH-exposed VF neurons (-58 ± 3 mV) after one hour. For both RPeD1 and VF neurons, the change in RMP between RA-exposed neurons and controls was found to be significantly different ($p>0.05$; Figure 3.9, B). These data corroborate previous observations (Dmetrichuk et al., 2006a) that exposure to RA hyperpolarizes the RMP of neurons. While hyperpolarization of the RMP may contribute to the decreased ability of RA-exposed neurons to fire spontaneous action potentials by bringing the cells further from impulse threshold, it is unlikely, that hyperpolarization accounts for the inability to induce action potentials with large electrical stimuli.

iv. Time course of RA-induced electrophysiological changes.

Previous work in our lab has shown that RA can enhance the electrical excitability of neurons over many days in culture (Dmetrichuk et al., 2006a). As such I next sought to determine whether the RA-induced changes in firing pattern, were transient or persistent. To this end, I first chose to determine whether the RA-induced effects lasted up to 3 hours. For these experiments I focused on VF neurons which were exposed to either 10μM RA (n=7) or 0.1% EtOH (vehicle) (n=9) for 3 hours prior to the start of recording. After the 3 hour exposure, neurons were impaled to determine if the ability to fire spontaneous or evoked action potentials had returned, and whether atypical impulse activity and action potential widening were still present.

Neurons exposed to RA or EtOH for 3 hours were capable of firing spontaneous or evoked action potentials throughout a 30 minute recording (Figure 3.10, A). Furthermore, no significant difference was found in the RMP between groups (RA: -66 ± 8 mV (n=7), EtOH:-68 ± 5mV (n=9)), suggesting that the RA-induced hyperpolarization of the RMP was a transient effect.

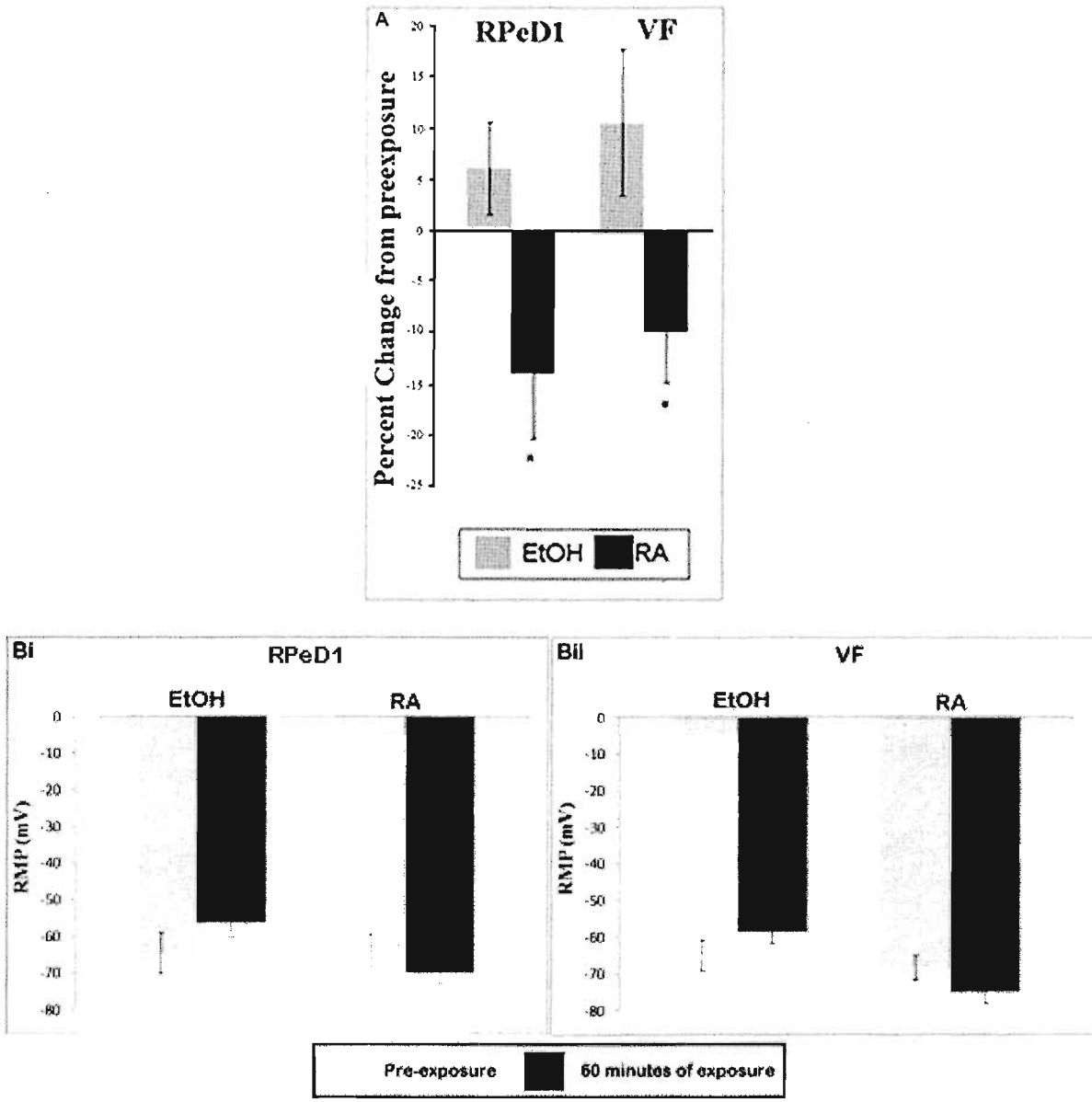


Figure 3.9. Acute exposure to RA causes hyperpolarization of RMP within the first hour of exposure. (A) Both RPeD1 and VF neurons exposed to RA (n=6 and 23, respectively) show a significant hyperpolarization of the RMP 60 minutes after RA exposure when compared to vehicle controls (n=6 and 19, respectively) within the same neuronal cell type. Values shown are calculated as a percent change from pre-exposure values (set at 0%) taken prior to the addition of RA or EtOH. Raw data values for RMP prior to and one hour after exposure to RA or EtOH for RPeD1 and VF neurons are shown in Bi and Bii, respectively. (*= $p<0.05$).

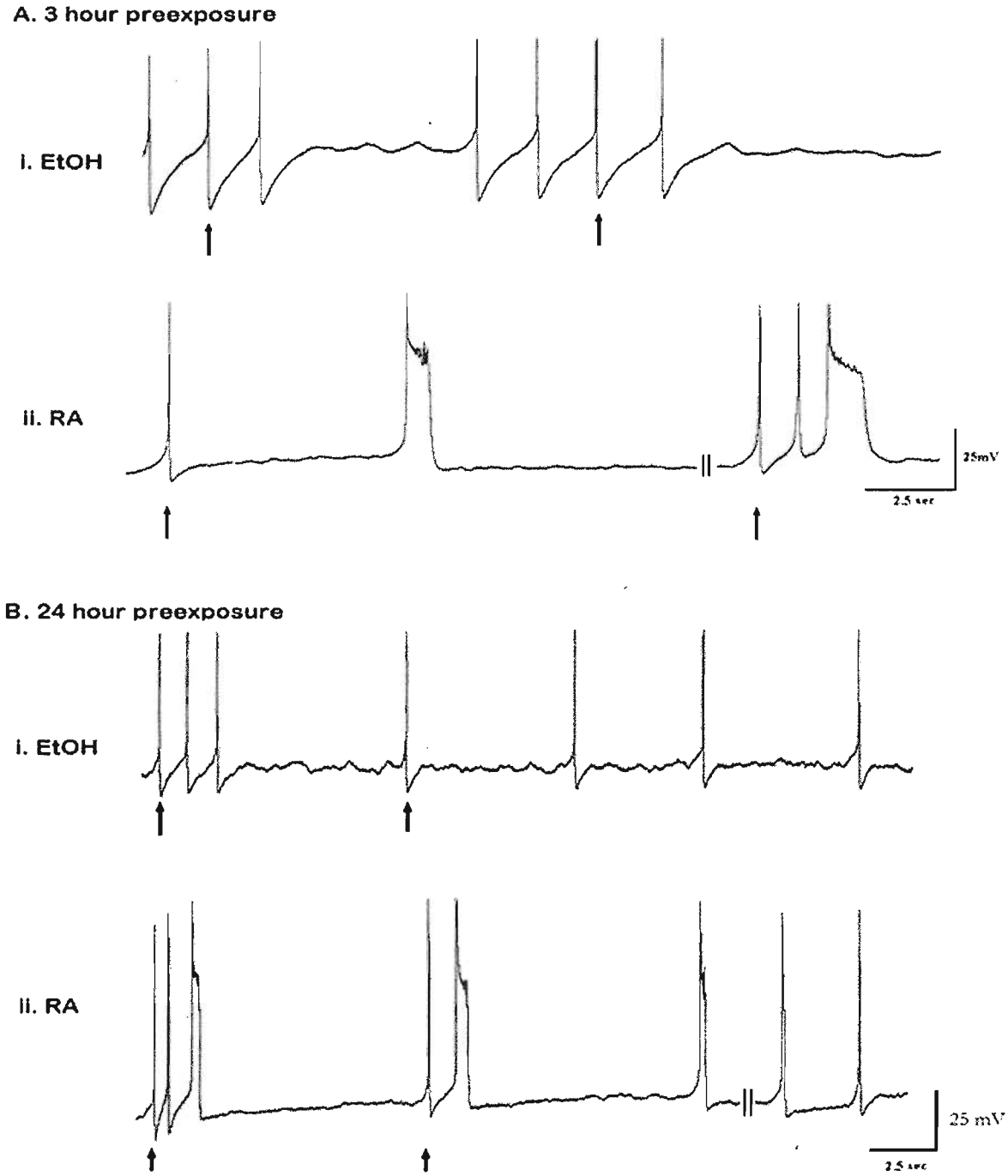


Figure 3.10. RA-induced atypical impulse activity persists for up to 24 hours. Representative examples of neurons exposed to RA or vehicle for 3 hours (A) and 24 hours (B). Neurons exposed to EtOH vehicle for 3 (Ai) or 24 (Bi) hours prior to the start of recording do not demonstrate qualitative changes in action potential shape and do not display atypical firing behaviors. Neurons exposed to 10μM RA for 3(Aii) or 24 (Bii) hours do not appear to show action potential widening when compared to control action potentials (arrows). Neurons exposed to RA for 3 (Aii) or 24 (Bii) hours do however still show the presence of atypical firing behaviors. || denotes a break in the recording.

Interestingly, despite no obvious action potential widening, atypical impulse activity was still observed (Figure 3.10). Approximately 86% (6/7) of neurons exposed to RA showed atypical firing behavior, whereas none of the control recordings (0/9) demonstrated such atypical activity. These data suggest that RA's effect on cell silencing is transient, and that the atypical firing pattern seen within the first hour of exposure is more persistent, lasting at least 3 hours after exposure to RA.

I next determined whether the atypical impulse activity persists for up to 24 hours after RA exposure. VF neurons were again exposed to 10μM RA or EtOH vehicle one day after plating but this exposure was now maintained for the next 24 hours, after which the cells were impaled and recordings were made. No significant difference was found in the RMP (t-test performed; RA: -69.9 ± 5.8 mV, EtOH 72.4 ± 3.0mV) or the ability of cells to fire spontaneous or induced action potentials in RA or EtOH-exposed neurons (Fisher exact test performed; RA: 12/14 cells could fire, EtOH: 12/12 cells could fire). Interestingly, the presence of atypical impulse activity was still observed in 71% (10/14) of RA-exposed neurons, whereas no such firing behavior was found in any of the control recordings (0/12; Figure 3.10, B). These data suggest that RA's effect on cell silencing is transient, and that the atypical impulse activity is indeed more persistent, as it is still observed 24 hours after exposure to RA.

III. RA-induced electrophysiological effects are dose-dependent and isomer-dependent.

Having shown above that acute exposure to RA results in qualitative changes in action potential shape and the firing properties of neurons as well as changes in the RMP, the next aim was to determine whether these responses were dose-dependent and/or isomer-dependent. The

experiments performed thus far in this thesis have utilized atRA, at a concentration of 10 μM, yet our lab has previously shown that *Lymnaea* neurons will respond to concentrations of atRA as low as 0.1 μM (Dmetrichuk et al., 2008; Farrar et al., 2009). Thus, I wanted to determine first whether the atRA-induced effects on cell firing were observed at lower concentrations. I also wanted to determine whether the RA-induced electrophysiological changes would occur in the presence of the 9*cis* isomer, indicating whether the response seen was isomer-specific. These studies again focused on the responses of the VF neurons only.

As shown in Figure 3.11, the RA-induced action potential widening was present in neurons exposed to 10μM atRA (n=28). This widening was also evident (but less dramatic) in cells exposed to 10μM 9*cis* RA (n=18). No widening was observed in neurons exposed to the lower dose (1μM) of atRA (n=22) or to the vehicle control, 0.01% EtOH (n=25). Interestingly, no significant differences were found in the RMP between any groups (data not shown; EtOH: n=19, 10μM atRA: n=23, 1μM atRA: n=20, 10μM 9*cis* RA: n=16).

Within the first hour of retinoid exposure, significantly more neurons exposed to 10μM atRA (n=28), but not 1μM atRA (n=22) or 10μM 9*cis* RA (n=18), showed atypical impulse activity than EtOH controls (n=25) (Figure 3.12, A). Additionally, significantly more neurons exposed to 10μM atRA showed atypical impulse activity when compared to 10μM 9*cis* RA. When examining the ability of RA to induce cell silencing, it was found that nearly 96% (24/25) of EtOH-exposed neurons were able to fire spontaneous or evoked action potentials 60 minutes after exposure (Figure 3.12, B). In stark contrast to these data, fewer than 4% (1/28) of 10μM atRA-exposed neurons were able to fire action potentials, either spontaneously or evoked, at the same time point. Interestingly, neurons exposed to 1μM atRA were all (22/22) able to fire

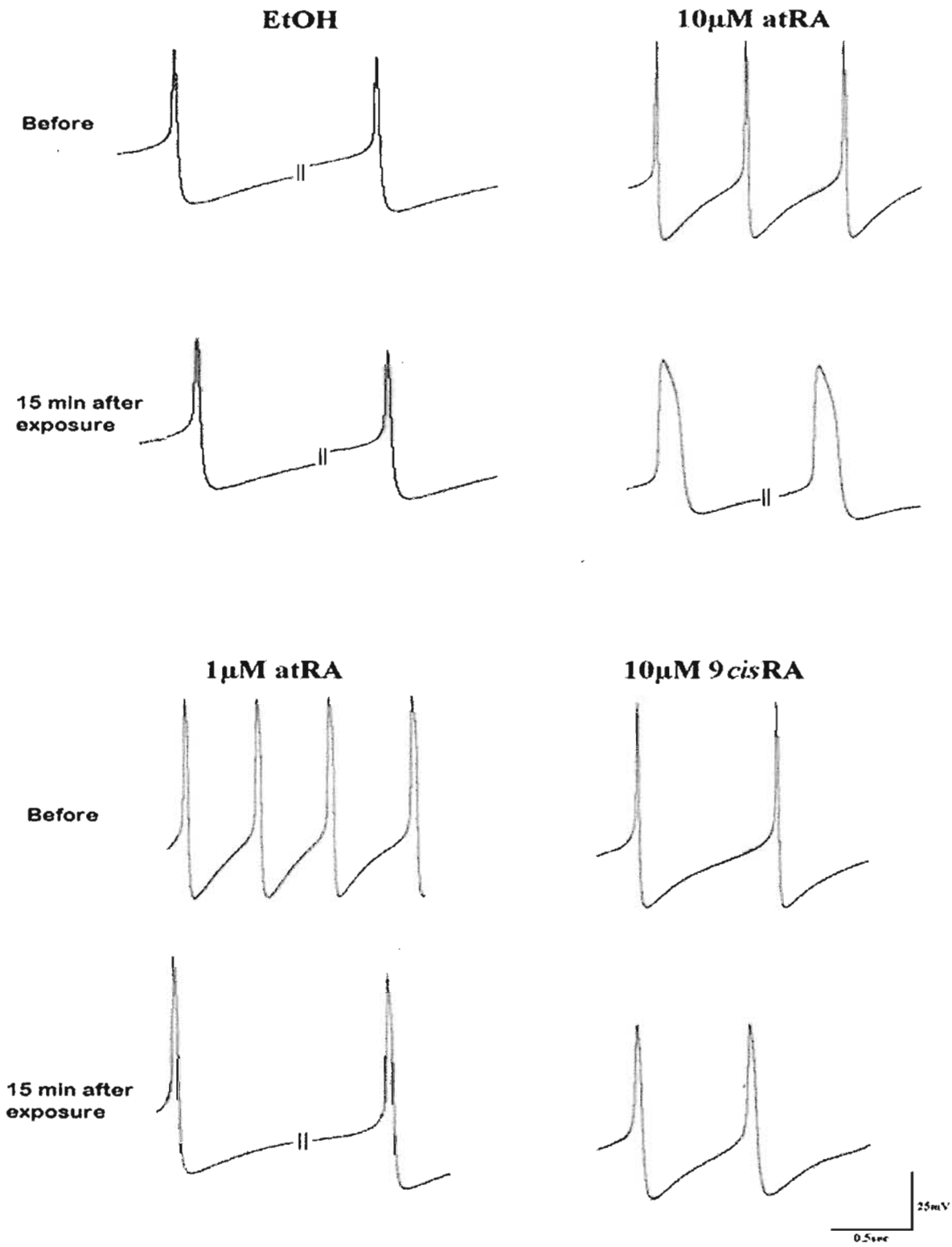


Figure 3.11. The RA-induced changes in spike waveform may be dose-dependent and isomer-specific in VF neurons. Exposure to 10μM atRA, but not 1μM atRA or EtOH, causes action potential widening. Widening is observed in 10μM 9*cis* RA-exposed neurons, however, the extent of widening is less dramatic than that seen in 10μM atRA-exposed neurons.

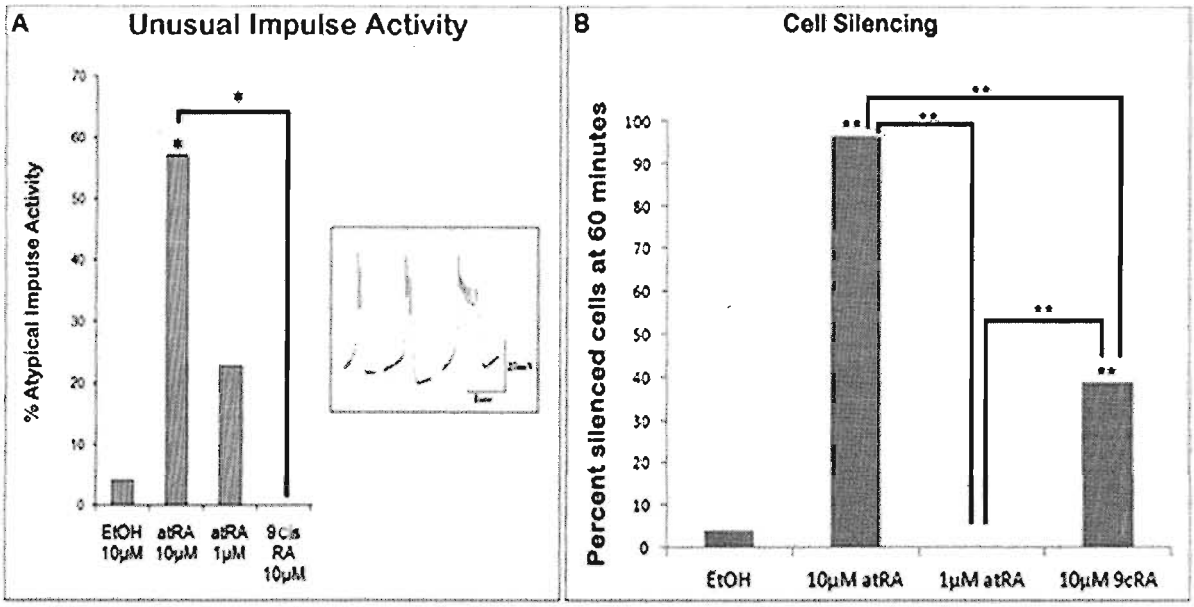


Figure 3.12. Dose and isomer-dependency of RA-induced changes in atypical impulse activity and cell silencing. A; More neurons showed atypical impulse activity if exposed to 10μM atRA (n=28), but not 1μM atRA (n=22) or 10μM 9*cis* RA (n=18) when compared to EtOH (n=25) 60 minutes after application. More neurons showed atypical impulse activity if exposed to 10μM atRA when compared to neurons exposed to 10μM 9*cis* RA. B; RA induced cell silencing was increased in 10μM atRA and 10μM 9*cis* RA-exposed neurons when compared to 1μM atRA and EtOH-exposed neurons. Asterix above bar represents a significant difference compared to EtOH controls. (*=$p<0.05$, **=$p<0.01$)

spontaneous or induced action potentials, suggesting that this dosage of atRA was unable to elicit RA-induced cell silencing. Lastly, approximately 40% (7/18) of neurons exposed to 10μM 9*cis* showed RA-induced cell silencing within 60 minutes of exposure. As shown in Figure 3.12, significant effects for both 10μM atRA and 10μM 9*cis* RA on cell silencing were found when compared to EtOH as well as 1μM atRA-exposed neurons, although the effects of 10μM atRA were significantly greater than those of 10μM 9*cis* RA. Taken together these data show an isomer dependency, with atRA having more dramatic effects than the same concentration of the 9*cis* isomer of RA. Additionally, the RA-induced effects appear to be dose-dependent, as a tenfold reduction in the concentration of atRA results in a decreased prevalence of atypical firing.

Due to the fact that many cells became silent after 35 minutes, I next examined changes in spike waveform, focusing on the 15 and 35 minute time points after retinoid application. Analysis of spike waveform at 15 minutes revealed that neurons exposed to 10μM atRA (n=22) had significantly increased half-amplitude durations compared to neurons exposed to 10μM 9*cis* RA (n=16), 1μM atRA (n=22), and EtOH controls (n=22; Figure 3.13, A). Additionally, 10μM 9*cis* RA-exposed neurons were found to have significantly increased half-amplitude durations than 1μM atRA-exposed neurons at this time point. Investigation of the half-amplitude duration at 35 minutes used fewer replicates due to the silencing effect of RA exposure. However, despite analysis of fewer cells, similar results were obtained. As before, 10μM atRA-exposed neurons (n=6) showed a significantly increased half-amplitude duration compared to 1μM atRA (n=8), 10μM 9*cis* RA (n=8) and EtOH controls (n=8) (Figure 3.13, B). Furthermore, at this time point, neurons exposed to 10μM 9*cis* RA were found to be significantly increased from EtOH controls. While these results are in agreement with the analysis performed at 15 minutes of RA treatment,

the RA-induced changes at 35 minutes were much more dramatic. During exposure to 10μM atRA, the half-amplitude duration increased by approximately 400% at 15 minutes and further increased to approximately 700% by 35 minutes of treatment. Additionally the effect of 9*cis* RA was significantly increased from EtOH controls at 35 minutes but not at 15 minutes. These data suggest that the RA-induced changes in action potential shape become more dramatic over time during the first 35 minutes of retinoid exposure.

In addition to the half-amplitude duration, the effect on decay time of 10μM atRA-exposed neurons was found to be significantly larger than that of 1μM atRA, 10μM 9*cis* RA and EtOH-groups 15 minutes after application (Figure 3.13, C). Similar results were obtained for the 35 minute time point and, like that of the half-amplitude duration, the effects were much more dramatic compared to the 15 minute time point. The 10μM atRA-induced increase in decay time was found to change from approximately 900% of baseline at the 15 minute time point to approximately 1600% of baseline at the 35 minute time point. No significant differences were found in the rise time of action potentials when compared to controls at either time point (Table 1). The input resistance one hour after retinoid exposure for each condition was also not significantly different from pre-exposure values (10μM atRA: n=26, 1μM atRA: n= 22, 10μM 9*cis* RA: n=16, EtOH: n=23). Interestingly, the peak to peak amplitude of action potentials from 10μM atRA-exposed neurons were found to be significantly reduced when compared to EtOH-exposed neurons (Table 1). However, this reduced amplitude was thought to be caused by the loss of the afterhyperpolarization in action potentials in 10μM atRA-exposed neurons (which is used in calculating the peak to peak amplitude). Continued analysis examining the amplitude of action potentials relative to the RMP revealed no significant differences (data not shown). Lastly, when comparing changes in the RMP one hour after retinoid exposure, no significant differences

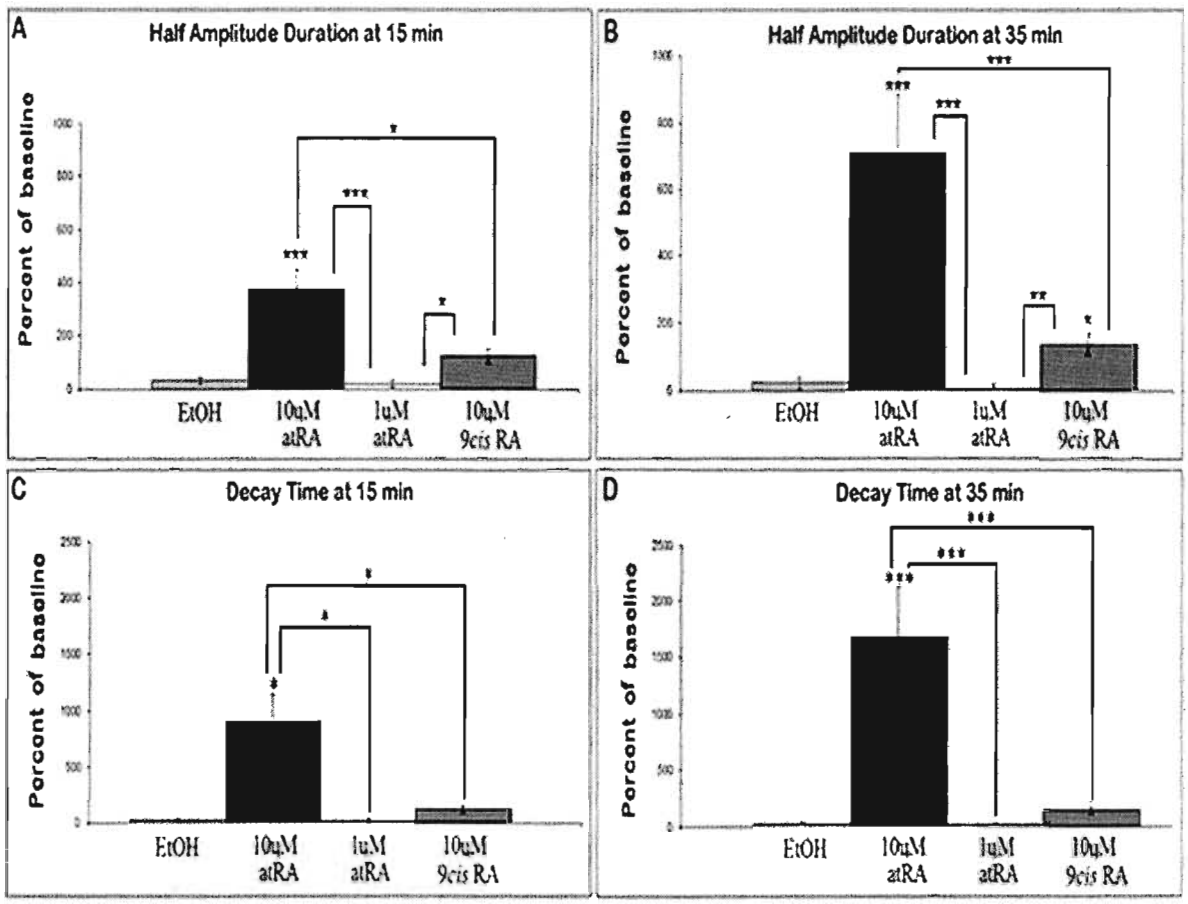


Figure 3.13. RA-induced changes in the half amplitude duration and decay time of neurons appear to be both dose and isomer-dependent. Half amplitude duration (A & B) and decay time (C & D) of neurons exposed to 10μM atRA, 1μM atRA , 10μM 9cis RA or EtOH, taken as a percent of baseline, set at 0%. Neurons exposed to 10μM atRA had significantly longer half amplitude duration (A) 15 and (B) 35 minutes after exposure when compared to 1μM atRA, 9*cis* RA and EtOH-exposed neurons. 9*cis* RA-exposed neurons were found to have significantly increased half amplitude durations when compared to controls at 35 minutes. Neurons exposed to 10μM atRA had significantly longer decay times (C) 15 and (D) 35 minutes after exposure, when compared to 1μM atRA, 10μM 9cis RA and EtOH -exposed neurons. Asterisk above bar indicates a significant difference compared to control condition. (* = $p<0.05$, ** = $p<0.01$ and ***=$p<0.001$). Number of replicates varied for each time point (see previous page).

Table 1 – Action potential waveform analysis and input resistance changes in RA exposed cells 35 minutes after application of retinoid or EtOH. 35 minutes post-retinoid application, 10μM atRA-exposed neurons were found to have significantly smaller peak to peak amplitude of action potentials when compared to 1μM atRA and EtOH-exposed neurons ($p<0.05$). In all other cases, no significant differences were found when compared to control condition. Peak to peak amplitude and rise time are expressed as a percent of baseline (set at 100%). Input resistance is expressed as a percent of baseline (set at 100%) after 60 minutes.

	35 minutes post exposure			
(% change of baseline)	**EtOH**	**10 μM atRA**	**1μM atRA**	**10 μM 9cis RA**
Peak to Peak Amplitude (%)	103.1 ± 6.3	73.0 ± 3.0*	108.1 ± 5.9	89.9 ± 2.8
Rise Time (%)	189.1 ± 36.2	402.8 ± 87.9	125.5 ± 24.5	213 ± 37.6
Input Resistance (%)	99.1 ± 8.8	113.1 ± 13.1	106.2 ± 9.0	114.3 ± 21.8

were found between any condition (10μM atRA: n=23, 1μM atRA: n= 20, 10μM 9*cis* RA: n=16, EtOH: n=19). These results, like that of the atypical firing behavior, show that the RA-induced changes in the firing properties of neurons was both dose-dependent and isomer-dependent.

Lastly, in addition to analyzing the waveform of single action potentials, I sought to determine whether frequency-dependent impulse broadening was altered in RA-exposed neurons. Frequency-dependent broadening is the widening of consecutive action potentials within a train of potentials, caused in part by a decrease in outward potassium current which leads to a prolonged falling phase of the action potential (Aldrich, Jr. et al., 1979). Changes in frequency-dependent broadening could provide insight into changes in ion channel activity during the action potential. Moreover, while the RA-induced effects described above show dramatic changes to single action potentials, these changes may be more pronounced during the firing of trains of action potentials. As shown in Figure 3.14, the frequency-dependent broadening of neurons exposed to 10μM atRA was found to be significantly greater than for EtOH controls. Additionally, 10μM atRA-exposed neurons showed significantly larger frequency-dependent broadening when compared to 1μM atRA exposed neurons. No difference was found between neurons exposed to 10μM 9*cis* RA and neurons exposed to 10μM atRA, 1μM atRA or EtOH control. Taken together these data show that single action potentials, as well as trains of action potentials are affected by acute exposure to RA.

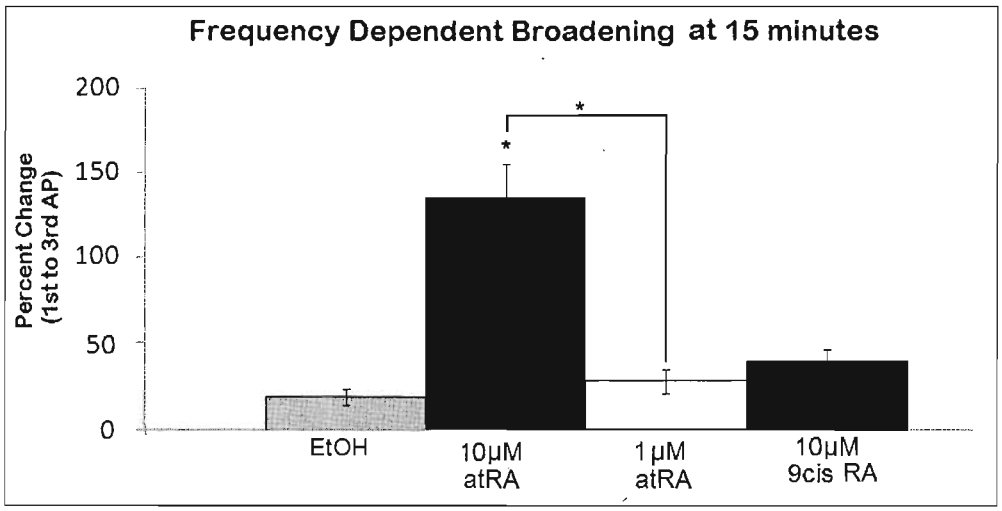


Figure 3.14. Frequency-dependent broadening is significantly increased in 10μM atRA-exposed neurons. 10μM atRA (n=17), but not 1μM atRA (n=18) or 10μM 9*cis* RA (n=14)-exposed neurons were found to have significantly increased frequency dependent broadening when compared to controls (n=17). 10μM atRA exposed neurons also had significantly larger frequency dependent broadening when compared to 1μM atRA. Data are expressed as a percent change from the first to third action potential (first action potential set at 0%). Asterisk above bar indicates a significant difference compared to control condition. (*=$p<0.05$)

IV. RA-induced effects are independent of electrical activity, trophic support and pre-exposure to low levels of RA

i. Electrical activity.

Previous work has shown that extension of neurites (Lautermilch and Spitzer, 2000; Cohan et al., 1987), growth cone motility (Ibarretxe et al., 2007a; McCobb et al., 1988; Cohan et al., 1987) and direction of growth cone extension (ie: attractive/repulsive; Ming et al, (2001)) can be modulated by changes in the electrical activity of neurons. In the following series of experiments I sought to determine whether RA's effects on cell firing may be modulated by the cell's electrical activity. More specifically, I examined whether silencing the electrical activity of neurons would delay the onset or reduce the magnitude of the RA-induced changes in firing pattern. These experiments were performed in cell culture with acute application of 10μM atRA, as in the previous section. Immediately after application of 10μM atRA, neurons were silenced by holding the resting membrane potential between -80 and -100mV for 30 minutes. If continual electrical activity indeed plays a role in the RA-induced effects, it would be expected that in the absence of such activity, the onset of the RA-induced effects would be delayed, or that the magnitude of these effects would be diminished, compared to RA-exposed neurons which were allowed to fire normally.

Neurons were hyperpolarized for the first 30 minutes after application of 10 μM atRA, described hereafter as "silenced" neurons, after which time they were allowed to return to rest and fire either spontaneous or evoked action potentials. This activity was then analyzed qualitatively for the presence of atypical firing behavior, and spike waveform was quantitatively analyzed at 35 minutes (5 minutes after silencing had ended) after retinoid exposure. As shown in Table 2, there was no significant difference in the number of neurons observed to exhibit atypical impulse

activity, and no difference in the spike waveform between neurons that were silenced (n=15) and neurons which were allowed to fire freely (n=16). Additionally, no difference was found in the input resistance from either group (Table 2). Lastly, silencing impulse activity did not affect the time course for the RA-induced cell silencing. In both cases, many neurons were still unable to fire spontaneous or evoked action potentials following exposure to RA. Specifically, 47% (7/15) of silenced and 56% (9/16) of unsilenced neurons were unable to fire action potentials at 35 minutes of RA exposure. By 60 minutes after RA application, 80% (12/15) of silenced and 81% (13/16) of control neurons were unable to fire. Overall, these data suggest that the firing activity of neurons had no impact on the time course or magnitude of RA's effects.

Interestingly, the RMP of silenced neurons was found to depolarize from -69 ± 4mV (measured prior to RA exposure) to -65 ± 2mV (measured 60 minutes after RA exposure), representing a 6% decrease over the duration of the recording. In contrast, unsilenced neurons exposed to RA hyperpolarized from -65 ± 8mV to -79 ± 8mV, representing a 21% increase. This difference was significantly different between groups (Figure 3.15, A; RA: n=8, RA + silencing: n=10). Lastly when comparing frequency-dependent broadening, silenced neurons (n=6) had significantly reduced broadening at the 35 minute time point compared to RA-exposed neurons that were allowed to fire freely (n=11) (Figure 3.15, B). These data suggest that a 30 minute hyperpolarization immediately after application of RA appears to partially prevent the effects of RA on the RMP as well as decrease the RA-induced increases in frequency-dependent broadening.

Table 2 – Action potential waveform analysis and input resistance changes in RA-exposed neurons which were silenced or allowed to fire freely. Atypical firing behavior (expressed as % observed) and action potential waveform analysis (expressed as a percent of baseline, set at 100%) in free firing (unsilenced) and silenced neurons exposed to 10μM RA. Input resistance is expressed as the percent of baseline levels (set at 100%) 60 minutes after exposure. In all cases, no differences were found (t-test performed for each parameter).

	10 μM RA	**10 μM RA + 30 min hyperpolarization**
Atypical impulse activity(% observed)	44	33
Peak to Peak Amplitude (%)	78.0 ± 6.3	84.4 ± 1.7
Rise Time (%)	237.7 ± 53.8	224.6 ± 59.3
Half-Amplitude Duration (%)	464.7 ± 138.1	419.6 ± 91.9
Decay Time (%)	875.3 ± 364.4	636.1 ± 123.1
Input Resistance (%)	141.1 ± 22.6	172.4 ± 71.0

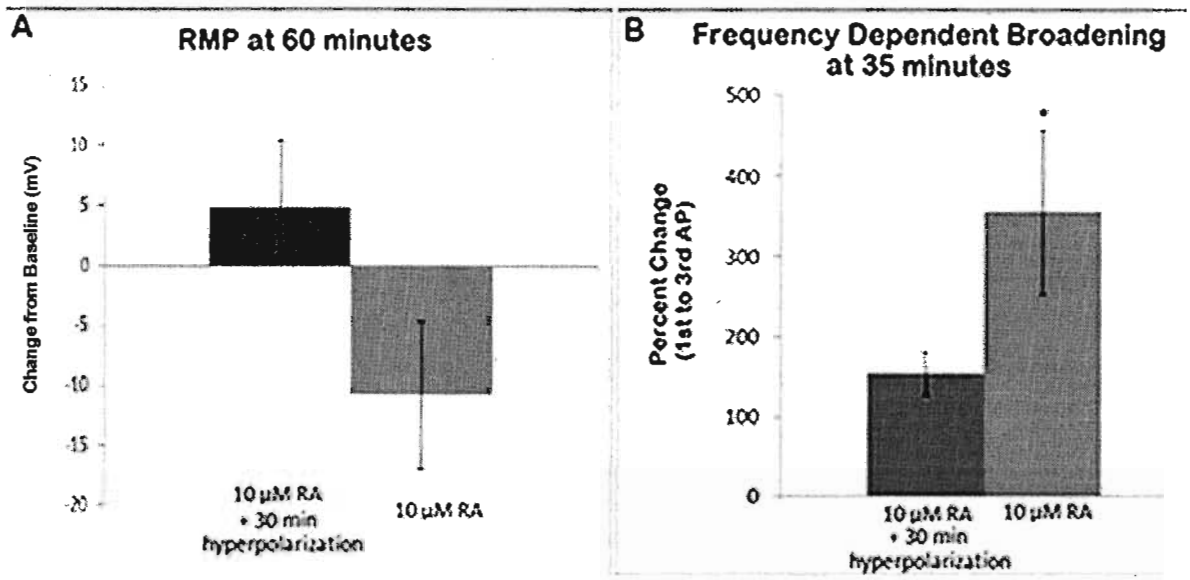


Figure 3.15. Hyperpolarization immediately after exposure to RA leads to a depolarization of the RMP 60 minutes after exposure and impairs RA-induced increases in frequency-dependent broadening. A: Neurons exposed to RA, followed by immediate silencing (n=10) show a depolarization of the RMP (become more positive), whereas neurons allowed to fire freely (n=8) show a significantly hyperpolarized RMP. B. Neurons which were forced to remain silent for the first 30 minutes following RA exposure (n=6) showed significantly reduced frequency-dependent broadening when compared to unsilenced neurons which were allowed to fire freely (n=11). (*=$p<0.05$).

ii. Provision of trophic support.

In the above experiments neurons were cultured in defined media (DM) for 24 hours prior to acute RA exposure without the addition of any trophic factors. Previous work has shown that neurons cultured under these conditions have impaired growth and electrical excitability as early as 2 days after culturing (Dmetrichuk et al., 2006). Additionally, trophic support has been shown to alter the electrical properties of neurons, both in terms of electrical excitability (Yamuy et al., 2000) and in the ability to reform synapses with target cells (Hamakawa et al., 1999). Thus, the RA-induced effects represented in this thesis may have occurred due to a compromised state of the cultured neurons lacking any trophic support over the first 24 hours. Therefore, I sought to determine whether provision of trophic support would alter the RA-induced effects. To this end I investigated whether this was the case by culturing neurons in conditioned media (CM), known to contain unidentified trophic factors (Wong et al., 1981) and comparing the effects of RA on cells cultured only in DM, which lacks trophic factors.

As shown in Table 3, the prevalence of atypical firing activity in response to acute RA exposure was not significantly different between neurons cultured in DM (n=8) or CM (n=10), suggesting that providing trophic support does not alter the RA-induced effects. Likewise, RA-induced cell silencing appeared to remain unchanged between each group, as 33% (2/9) of neurons cultured in DM and 20% (2/10) of neurons cultured in CM were silenced within 60 minutes of RA exposure. Similarly, analysis of spike waveform 15 and 35 minutes after retinoid exposure revealed that there was no significant difference in the half-amplitude duration or decay time of neurons cultured in DM or CM (Table 3). Similarly, no difference was found in the peak to peak amplitude or rise time of neurons cultured in DM compared to neurons cultured in CM. Furthermore, no difference was found when comparing the input resistance between either

condition (DM: n=7, CM: n=8) and no difference was found in the RMP between neurons cultured in DM or CM and then exposed to RA for 60 minutes (CM: n=8, DM: n=6). Lastly, no difference was observed when comparing the frequency-dependent broadening of either condition (DM (n=6) or CM (n=7)). Taken together these data provide substantial evidence suggesting that the presence of trophic support did not alter the RA-induced effects in producing atypical firing behavior, cell silencing or changes in action potential waveform. Thus, it is unlikely that the RA-induced effects seen are caused by a compromised state of the neurons due to the absence of trophic support for the first 24 hours in culture.

iii. Pre-exposure to low levels of RA.

DM not only lacks trophic factors but also lacks RA. The CNS transcriptome of *Lymnaea* has been shown to contain the metabolic machinery necessary for the synthesis of RA from dietary retinol (Feng et al., 2009). Thus, culturing in the absence of RA or metabolic precursors to RA may pose as a stressor and alter the response of neurons to RA. In other words, the inability to synthesize RA due to the lack of metabolic precursors may have compromised the health of cultured neurons and, thus, alter the response of these neurons to RA. In an attempt to elucidate whether this is the case, I next investigated whether pre-exposure to the estimated endogenous level of RA (~0.1 μM) (Dmetrichuk et al., 2008), would alter the acute response to RA. These experiments were conducted exactly as in the previous section, except that immediately after plating neurons into culture dishes, 0.1 μM atRA or 0.001% EtOH (as a control) was added to the media. Neurons were incubated until the following day, at which point they were acutely exposed to the higher level of 10μM atRA as performed previously. In these experiments a group of neurons which did not receive pre-exposure to RA or EtOH was used as a positive control to test their response to 10 μM atRA (referred to as control RA).

Table 3 – Trophic support does not appear to alter RA's effects on firing pattern. Representative spike waveform data displayed for 35 minutes after retinoid exposure (expressed as a percent of baseline, set at 100%). Input resistance is shown for 60 minutes after retinoid exposure displayed as a percent change of baseline. RMP displayed as the percent of baseline 60 minutes after RA exposure, set at 100%. Frequency dependent broadening displayed as the percent of the 3rd spike (relative to the first action potential, set at 100%) within an action potential train 15 minutes after retinoid exposure. In all cases no significant differences were found (t-test between CM and DM conditions for each parameter).

	DM + 10 μM RA	**CM + 10 μM RA**
Atypical impulse activity(% observed)	100	80
Peak to Peak Amplitude (%)	88.5 ± 4.2	94.7 ± 3.6
Rise Time (%)	181.8 ± 39.3	104.9 ± 13.5
Half Amplitude Duration (%)	212.2 ± 42.7	276.9 ± 71.5
Decay Time (%)	671.8 ± 276.1	845.1 ± 403.5
Input Resistance (%)	81.9 ± 13.1	135.4 ± 20.0
RMP (%)	106.8 ± 7.9	105.5 ± 8.8
Frequency Dependent Broadening (%)	206.5 ± 47.5	266.3 ± 46.7

No differences were found between the RA pre-exposed (n=11) or vehicle pre-exposed (n=12) groups and control RA (with no pre-exposure; n=13) when analyzing atypical impulse activity and spike waveform both at 15 and 35 minutes after RA exposure (Figure 3.16: A-D). Additionally, no significant differences were found when examining the RA-induced cell silencing. Within 35 minutes of exposure to 10 μM RA, 25% (3/12) of EtOH pre-exposed and 45% (5/11) of RA pre-exposed neurons were unable to fire spontaneous or evoked action potentials. 60 minutes after retinoid exposure the number of silenced cells increased to 33% (4/12) for EtOH-exposed and 54% (6/11) of RA pre-exposed neurons. Similarly, 39% of neurons which did not receive any pre-exposure but were acutely exposed to 10 μM RA were found to be silent at both time points. Additionally, in all cases, the input resistance of neurons 60 minutes after exposure were not significantly different compared to pre-exposure values within each group (control RA: n=13, RA pre-exposure: n=9, EtOH pre-exposure: n=12) (Table 4). Furthermore, no change in the RMP was found (control RA: n=8, RA pre-exposure: n=7, EtOH pre-exposure: n=11), and frequency-dependent impulse broadening was not significantly different between any groups, as shown in Table 4 (control RA: n=11, RA pre-exposure: n=7, EtOH pre-exposure: n=7). Taken together, these data suggest that pre-exposure to a lower, physiologically relevant concentration of RA does not alter the neuron's response to higher concentrations of RA.

V. RA-induced effects occur in an isolated neurite lacking a cell body.

During the course of the experiments conducted in this chapter, one RPeD1 neuron was cultured with a long portion of its original primary neurite intact. This section of "original" neurite was sufficiently large, in this one condition, to permit transection of the neurite from the cell body and then to make an electrophysiological recording directly from both the soma (used as a

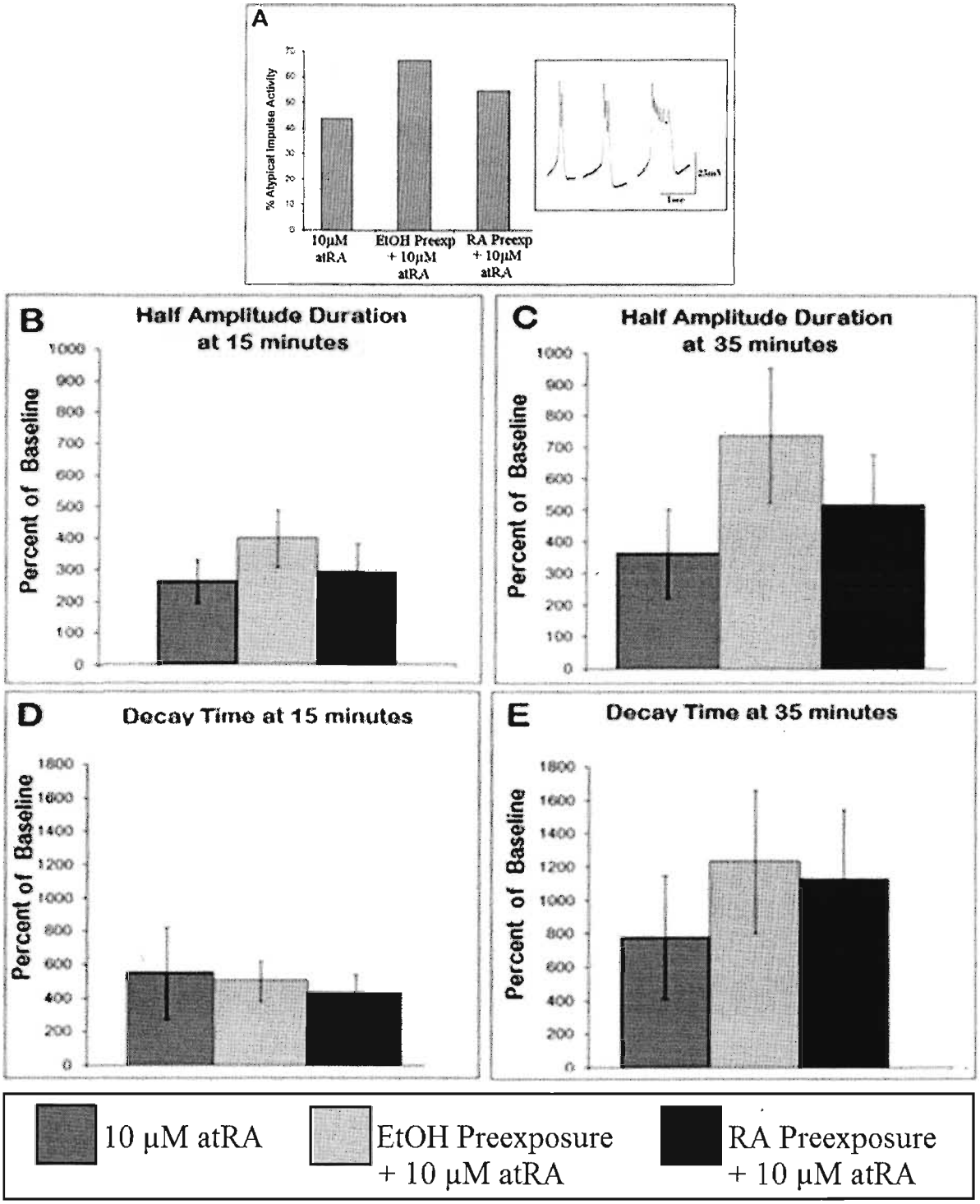


Figure 3.16. Pre-exposure to low levels of RA has no effect on acute response to 10 μM RA. Pre-exposure to 0.1 μM atRA does not alter the RA-induced increases in atypical firing behavior, half amplitude duration or decay time. (A) Neurons pre-exposed to 0.1 μM atRA or EtOH did not show significantly less atypical impulse activitywhen compared to RA controls without pre-exposure. The half amplitude duration (B) 15 and (C) 35 minutes and decay time (D) 15 and (E) 35 minutes after RA exposure was not significantly different between groups. Data are expressed as a percent of baseline (set at 0%).

Table 4 – Pre-exposure to low levels of RA does not appear to alter acute RA-induced changes in firing pattern and spike waveform. Representative data for peak to peak amplitude and rise time of action potentials displayed for 35 minutes after retinoid exposure, taken as a percent of baseline, set at 100%. Input resistance is shown for 60 minutes after retinoid exposure displayed as a percent of baseline, set at 100%. RMP is shown as the percent of baseline one hour after RA-exposure, set at 100%. Frequency-dependent broadening displayed as the percent of the 3rd spike (relative to the first spike) within an action potential train 15 minutes after retinoid exposure. In all cases no differences were found. ANOVA performed for all comparisons

	10 µM atRA	**EtOH Pre-exposure + RA**	**0.1 µM atRA Pre-exposure + RA**
Peak to Peak Amplitude (%)	92.3 ± 4.6	84.9 ± 3.5	80.0 ± 5.7
Rise Time (%)	108.8 ± 15.8	161.6 ± 35.1	289.7 ± 88.2
Input Resistance (%)	141.1 ± 22.6	128.9 ± 20.5	123.2 ± 16.3
RMP (%)	122.4 ± 11.9	118.2 ± 3.9	115.9 ± 13.0
Frequency Dependent Broadening (%)	206.5 ± 47.5	154.4 ±27.4	168.7 ± 54.1

positive control) and isolated neurite simultaneously. My aim was to determine whether RA could exert its electrophysiological effects in the absence of the cell body and thus in the absence of any classical transcriptional activation. Previous evidence from our lab has shown that RA can elicit some effects in the absence of a cell body and, thus, independently of transcriptional activation (Carter et al., 2010; Farrar et al., 2009). Specifically, these studies showed that RA can induce growth cone turning in cultured neurites which have been transected from the soma. In this experiment, the neurite was transected and allowed to recover for 10 minutes (Figure 3.17, A-C), after which it was exposed to RA and recordings were made. Due to the difficulties associated with stable recording from neurites, RA was added immediately prior to the insertion of the electrodes into the neurite and cell body. As shown in Figure 3.17, D, when compared to the firing pattern 5 minutes after RA exposure (used as a control time point), both soma and neurite show a marked change in the firing pattern 45 minutes after RA exposure. Importantly, the neurite showed RA-induced action potential widening, the presence of atypical impulse activity, reduced afterhyperpolarization, and became silent approximately 50 minutes after RA exposure. These data show that RA can induce similar changes in the firing properties of an isolated neurite as typically seen in the intact cell body. Thus RA does not appear to require the presence of the soma to elicit its effects. Attempts to repeat these experiments were unsuccessful as the original neurite process often retracted after plating of the cells into culture dishes, and was not sufficiently large to allow for transection the following day.

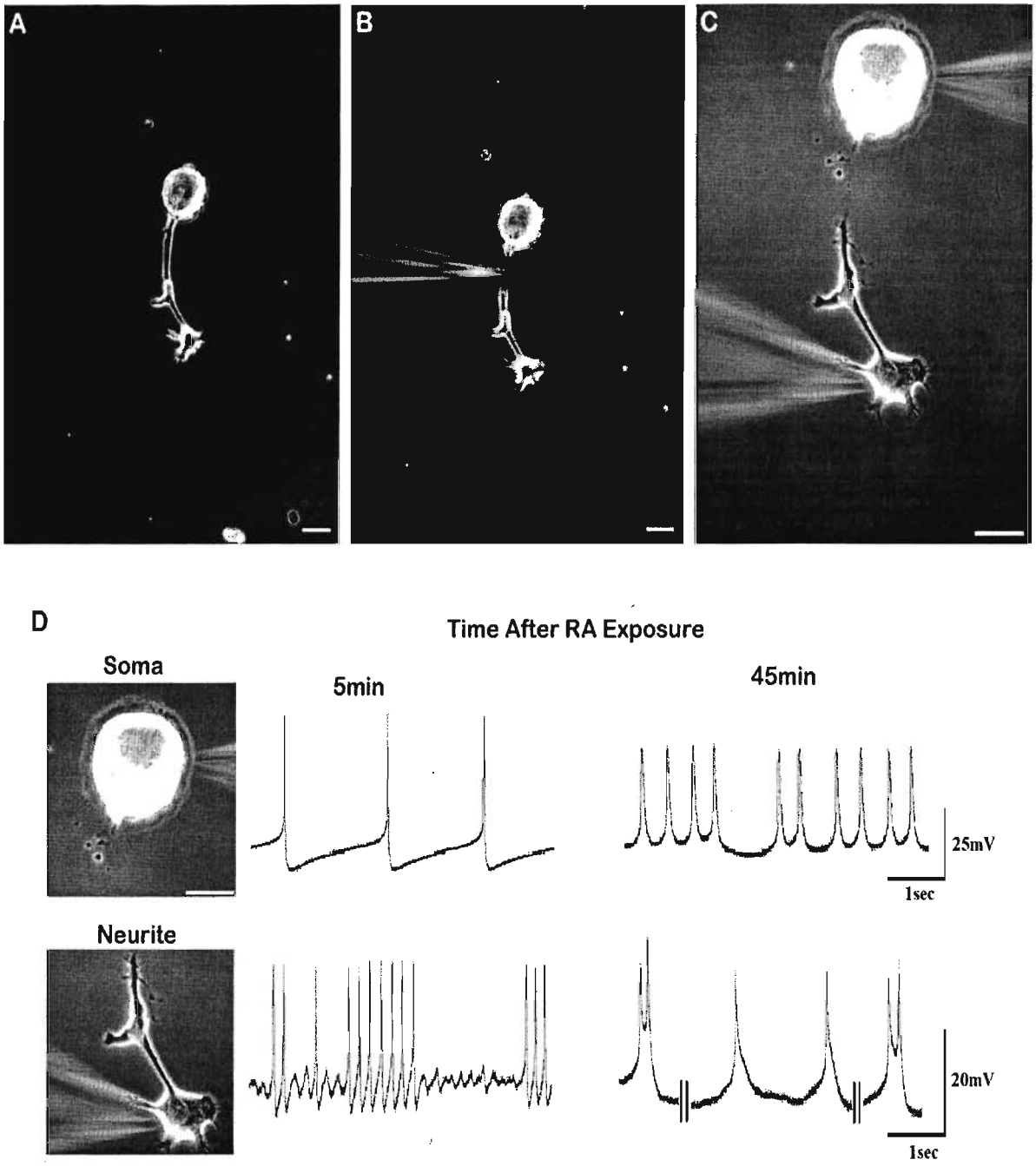


Figure 3.17. RA-induced firing changes occur in an isolated neurite lacking a cell body. Image of a cultured RPeD1 neuron possessing a large neurite (A), which was isolated and removed from the cell body via transection with a glass electrode (B). Approximately 10 minutes after transection, the neurite had partially retracted away from the cut site, showing both the soma and neurite as separate entities, which were simultaneously recorded from (C). D: simultaneous electrophysiological recordings from both the soma (upper traces) and neurite (lower traces) after exposure to RA. Within 45 minutes of exposure to RA, both the soma and neurite show altered firing properties, displaying action potential widening, reduced afterhyperpolarization and the presence of atypical impulse activity, when compared to recordings taken 5 minutes after RA-exposure. The time course of the RA-induced effects in both soma and neurite appeared to be similar. Scale bar in all cases: 100μm. || denotes a break in the recording.

3.05 – Discussion

Very little work has previously been performed examining RA's ability to elicit an electrophysiological change in neurons. While some work has examined RA's ability to elicit change over many days in culture (Arcangeli et al., 1998; Tonini et al., 1999), presumably through RA's influence on gene transcription, few studies have examined whether RA can rapidly modify a cell's electrical properties. Indeed, to the best of my knowledge only two studies have documented the ability of RA to elicit a rapid electrophysiological change. First, in studies performed by Xaio et al. (1998), RA was shown to cause the rapid inhibition of sodium currents in cultured human embryonic kidney cells. Secondly, work performed by Zhang and McMahon (2000) demonstrated that RA can rapidly decrease electrical coupling in retinal neurons of the striped hybrid bass. However, neither of these studies has investigated whether RA has the capacity to alter the firing properties of adult regenerating neurons. The main aim of the present series of experiments was to characterize any RA-induced changes in firing pattern. Given that RA can enhance and direct neurite outgrowth, processes which have been shown to be modulated by electrical activity (Ibarretxe et al., 2007a; Ming et al., 2001; Lautermilch and Spitzer, 2000), the results presented in this chapter may provide insight into elucidating how RA elicits its regenerative effects.

The work discussed here has shown, for the first time in any animal species, that the application of RA can elicit dramatic changes in the firing properties of adult regenerating neurons. Within this chapter I have characterized three main effects on firing pattern induced by RA-exposure. First, application of RA caused the presence of atypical impulse activity. That is, RA-exposed neurons exhibited dramatic bursting behavior, as well as plateau potentials and spike doublets. Secondly, RA application produced changes in the action potential waveform, increasing the

half-amplitude duration and decay time. Lastly, RA application caused a transient silencing effect on cell firing. This silencing was transient, as neurons exposed to RA for 3 hours regained the ability to fire. Interestingly, neurons which have been exposed to RA for 3 hours display single action potentials comparable to those of controls, with no action potential widening. The presence of atypical impulse activity however, is still observed and can also be observed for at least 24 hours after RA exposure. These data suggest that the presence of atypical impulse activity may be independent of changes in spike waveform. This also suggests that acute RA exposure induces both a short-term effect; action potential widening and cell silencing (lasting at least one hour), and a long term effect, the presence of atypical impulse activity (lasting at least 24 hours).

Interestingly, similar electrophysiological changes to those documented here in response to RA have been reported in damaged neural tissue. More specifically, work utilizing reticulospinal neurons from the lamprey has shown that transected neurons change their firing pattern from smooth trains of action potentials to short repetitive bursts (McClellan et al., 2008). In the studies performed here, RA exposure did not consistently induce bursting activity, however when bursting was observed it was typically repetitive with comparable inter-burst intervals and fairly consistent burst durations. Thus, RA exposure appears to induce changes in the firing activity of neurons similar to those induced by neural injury. McClellan et al. (2008) suggested that the alterations in firing pattern and action potential shape may act to promote a favorable intracellular environment, potentially containing less intracellular calcium, for neurons to regenerate. Other work has shown that action potential firing (Ibarretxe et al., 2007b) as well as calcium transients induced by spontaneous activity (Lautermilch and Spitzer, 2000) can result in calcium-mediated growth cone stalling. Similarly, work in *Helisoma* has shown that increased

electrical activity and exposure to serotonin, both of which inhibit outgrowth, cause transient increases in intracellular calcium (Cohan et al., 1987; McCobb et al., 1988). This work, thus, strengthens the above notion that reduced firing activity and altered intracellular calcium levels may be required to promote the induction of neurite outgrowth. My current findings add further support, as RA-exposure results in the transient cell silencing of firing activity lasting up to 3 hours after exposure. Since reduced action potential firing would reduce calcium influx, it may be possible that the RA-induced cell silencing serves to reduce intracellular calcium levels so as to promote the initiation of neurite outgrowth. Thus RA's ability to induce and enhance neurite outgrowth may come from the ability to transiently silence cell activity and allow for a favorable intracellular environment to potentially promote a regenerative response. Continued studies investigating whether this may be the case, are required to validate this speculation.

With the assumption that the RA-induced cell silencing is a physiologically relevant response to promote the induction of neurite outgrowth, my data show that this is a short-lived effect, as firing activity is restored within 3 hours of RA exposure. This result is potentially explained by work that has shown that the electrical activity of neurons can dramatically alter not only the sensitivity of growth cones to guidance molecules, but can also determine the type of response (ie: attractive/repulsive) to local cues (Ming et al., 2001) and modulate the extension of neurites (Lautermilch and Spitzer, 2000). Thus, while it has been suggested that reduced firing activity is required to promote regeneration (McClellan et al., 2008), it would appear that actively extending neurites may require continued firing activity to modulate their guidance. Thus, after the induction of neurite outgrowth (ie: sprouting) the gradual return of firing activity would be expected to aid in the guidance of the newly formed neurites to their targets. While substantial work would be required to validate this claim, it is tempting to speculate that the transient cell

silencing effect caused by RA exposure, lasting up to 3 hours, acts to promote the induction of neurite outgrowth, after which cell firing returns to aid in the guidance of the newly formed neurites. At this time, the continued atypical firing activity which is seen for at least the first 24 hours after RA exposure remains to be explained.

Surprisingly, RPeD1 neurons exhibited electrophysiological responses to acute RA exposure in culture, but not in the intact CNS. In culture, these neurons showed the same RA-induced changes in firing pattern seen in VF neurons: atypical impulse activity, action potential widening and eventual cell silencing. My work investigating acute RA exposure in the whole CNS, however, showed that RPeD1 neurons did not appear to be responsive to RA application while VF neurons showed similar responses as seen in culture. Moreover VF, but not RPeD1 neurons were more responsive to acute RA exposure if a regeneration situation (a nerve-crush injury) had been elicited for 24 hours. It is interesting that RPeD1 neurons did not exhibit electrophysiological responses to RA exposure in the whole CNS (at least at the level of the soma) but did respond in culture. I had hypothesized that isolation of the CNS and application of a nerve-crush injury would initiate a regenerative response, and that this would be similar in both whole CNS and cell culture conditions. The current results may suggest that removal of the RPeD1 soma from the CNS into culture conditions may initiate a different regenerative response, as RPeD1 now lacks all synaptic inputs. My previous work (Chapter 2) utilizing an *in situ* preparation in which RPeD1 was crushed may still have allowed a number of synaptic connections to be maintained. Specifically, the fine neurite processes in the pedal ganglion, as well as a large portion of RPeD1's main axon were left intact in the nerve-crushed CNS. Thus, this condition may represent a comparatively minor injury environment compared to cell culture conditions, where RPeD1 is completely severed from all of its processes and associated synaptic

input. Thus, cell culture conditions may cause a different regenerative response, one that is responsive to RA, owing to the increased severity of neural damage. Thus, the differing levels of neural trauma inflicted on RPeD1 may explain why the differing response to RA across these two experimental preparations may be different. Alternatively, there may have been an electrophysiological response in RPeD1 to RA in the whole CNS, but the site of action *in situ* may have been distant from the soma and thus not able to be recorded from at the level of the soma. My data are in support of this notion, as VF cells received a nerve-crush adjacent to the ganglia containing VF somata, which would have likely caused the degeneration of VF axons almost completely to the soma. RPeD1 neurons however, did not receive a nerve-crush which caused the degeneration of its axon back to the soma. Thus, if RA elicits a response at the site of nerve damage, it is likely that this response would be recorded in VF somata (due to their close proximity to the damage site), but not necessarily from RPeD1 somata. Continued studies would be required to validate this hypothesis.

Additionally, I have shown that the RA-induced changes appear to be dose-dependent, as a 10 fold reduction in the concentration of atRA resulted in significantly reduced atypical firing activity, RA-induced cell silencing and reduced alterations in spike waveform when compared to the higher dose of 10μM atRA. While RA concentrations of 1μM atRA (or lower) are typically considered to be physiologically relevant (Dmetrichuk et al., 2008; Dmetrichuk et al., 2006), my data suggest that at this concentration, RA does not elicit significant changes in the firing patterns of neurons. As a higher dose of RA can induce dramatic changes in firing properties, these data raise the question of whether RA is ubiquitously distributed within the adult CNS or localized to specific regions. It may be possible that local increases in RA concentration either in microdomains within neural networks (such as at synapses) or in the vicinity of specific

neurons, lead to specific targeted changes in the firing pattern of adult neurons. RA is known to establish a steep concentration gradient within developing tissue (Dolle, 2009; Maden, 2007), and thus the possibility of transiently producing local areas of RA at high concentrations in adult tissue is not without precedent. Furthermore, upon neural trauma the metabolic machinery required to synthesize and transport RA, have been shown to be dramatically upregulated at the mRNA and protein levels (Zhelyaznik et al., 2003; Nagashima et al., 2009; Reimer et al., 2009), suggesting that RA production may be increased locally in response to injury. Thus, it may be possible that RA concentrations dramatically increase at specific targeted sites of neural damage, rather than ubiquitously within the entire CNS.

Currently it is unclear whether the effects of 10 μM atRA shown here represent physiologically relevant effects or whether these are "non-physiological" or pathological responses, as typically RA concentrations up to the nanomolar range have been thought to be physiologically relevant (Scadding and Maden, 1994; Chen et al., 1994). Thus, the response to RA documented here utilizing a micromolar concentration may be the result of RA toxicity. If this is indeed the case, then my data may have implications for the clinical use of retinoids in (for example) cancer and dermatology. The use of RA in the treatment of promyelocytic leukemia has been known to increase the risk of patients developing retinoic acid syndrome, which can ultimately lead to death (Patatanian and Thompson, 2008). Similarly, isoretinoin, an RA analogue used to treat acne, has been suggested to lead to increased rates of suicide (Hull and D'Arcy, 2003). In both cases it would appear that excessive use of RA as a therapeutic agent can have adverse effects. Thus, the ability for RA to alter the activity of adult neurons in a pathological manner must be taken into consideration. While it is possible that the effects shown in the current studies represent pathological responses to RA, I feel this is unlikely. First, studies performed in other

animal species have used concentrations ranging from 10μM (Liao et al., 2004) to as has high as 100μM (White et al., 2007) without any obvious neuropathological effects. Moreover, 10μM atRA, while inducing limb deformities, allows for the otherwise normal development of zebrafish embryos (Vandersea et al., 1998). Secondly, there have been reported cases where micromolar levels of RA have been found *in vivo* (De et al., 1992) and where micromolar levels of RA are required to elicit cell differentiation (Quenech'Du et al., 1998). Thus the notion that RA can be utilized endogenously at micromolar levels to elicit its effects is not without precedent. Lastly, my work has shown that neurons cultured in the presence of 10μM atRA for 24 hours maintain the ability to fire seemingly normal action potentials and maintain RMPs that are comparable to controls. These data strongly suggest that neurons can maintain their normal electrical properties despite prolonged exposure to10μM RA. Moreover, neurons in the present work are cultured in DM, lacking any tropic support, and thus are arguably more susceptible to toxicological effects associated with exposure to excessive concentrations of a chemical. Given that the RA-exposed neurons are comparable to controls (other than the presence of RA-induced atypical impulse activity) these data would suggest that a 10μM concentration of atRA may not be inducing pathological effects and that the results obtained here are likely to be physiologically relevant. Further study will be required to validate this claim.

In addition to dose-dependent effects, my data also suggest that the atRA isomer elicits significantly more pronounced atypical firing activity, cell silencing and spike waveform modifications compared to the same concentration of the 9*cis* RA isomer. This result is particularly interesting, as 9*cis* RA has often been found to have substantially greater (more than 10x) potency than the atRA isomer in vertebrate systems (Han et al., 1995; Thaller et al., 1993), but work performed previously in *Lymnaea* suggested that both isomers elicit similar effects.

More specifically, both isomers were shown to induce, enhance and direct neurite outgrowth with no clear indication as to which isomer (if any) is more potent (Dmetrichuk et al., 2008). Interestingly this work has shown that, while not significantly different, neurons were 20% more likely to extend neurites if exposed to atRA than the same concentration of 9cis RA. Thus atRA, in some cases, might be more effective than 9*cis* RA in inducing regenerative responses; however continued studies are required to validate this claim. My current data show, for the first time in *Lymnaea,* that isomers of RA may act differently, and in particular suggest that the atRA isomer is able to elicit significantly more dramatic effects than that of the 9*cis* RA isomer. As discussed in the following sections, continued efforts to elucidate how this may occur (eg: such as through the differential activation of retinoid receptors) within the CNS were performed as described in the following chapter. These current data represent the first steps taken towards characterizing the rapid RA-induced change in electrophysiological properties of neurons.

Lastly, there remains the possibility that RA may elicit its effects via non-specific actions on lipid membranes. There is work documenting RA's ability to destabilize membranes in rats (Tiwari et al., 2007; Meeks et al., 1981). This work has shown that RA, in micromolar concentrations, can destabilize membranes through an unknown mechanism, leading to lipid breakdown which can potentially lead to ion concentration imbalances and thus affect the electrical properties of neurons. Interestingly, this work showed that both atRA and 13*cis* RA elicited a similar level of membrane destabilization, suggesting that both the *trans* as well as *cis* isomers of RA may have similar non-specific effects on the membrane (Meeks et al., 1981). Assuming that all isomers of RA can indeed destabilize membranes equally, my data would argue against a non-specific effect of RA on membranes, as I clearly show that atRA exerts effects on the electrical properties of neurons that are not evident with the same concentration of

the 9*cis* RA isomer. Furthermore, Meeks et al. (1981) showed that atRA induces substantial membrane destabilization at concentrations as low as 1 μM. Thus if RA destabilized membranes in my current work I would have expected to see a significant difference in neurons exposed to 1 μM atRA compared to control. This is not the case as only cells exposed to 10 μM RA show changes in firing pattern. Lastly, if RA was acting by destabilizing membranes one would expect an accompanying change in the input resistance of neurons exposed to RA. My data do not support this hypothesis and show that the input resistance of RA-exposed neurons remains unchanged, both with respect to time as well as to the control condition. Thus, the dose-dependency and isomer-dependency of the RA-induced changes in firing pattern, as well as the unaffected input resistance of neurons would suggest that RA may not be having non-specific effects on lipid membranes.

Having characterized an acute response to RA in culture, an obvious question for continued work would be to determine how RA elicits these effects. Currently it is unclear whether RA acts directly or indirectly on, for example, retinoid receptors, ion channels or second messenger systems to produce the required changes in firing properties. There is a growing body of literature that documents the ability of RA to elicit its effects through a myriad of signaling pathways, extending well beyond that of the classical retinoid signaling pathway which exclusively utilizes transcriptional activation. My data show clear significant differences in action potential waveform in as little as 15 minutes after RA application. This time course does not necessarily rule out the possibility that RA may be acting to alter gene transcription to elicit the effects shown here. However I have provided evidence utilizing an isolated neurite that RA has the ability to elicit changes in firing properties in the absence of the cell body and nucleus. This preliminary evidence suggests a local response to RA and, thus, strongly suggests that RA

may be operating in a non-genomic fashion. It has been previously shown in *Lymnaea* that the RXR has the capacity to operate in a non-genomic fashion to direct growth cone guidance (Farrar et al., 2009), and thus the possibility that the retinoid receptors may play a similar non-genomic role in the results shown here is not without merit. In the next chapter, I will investigate the possibility that RA exerts its effects on the electrophysiological properties of neurons either via direct actions on intracellular pathways or involvement of retinoid receptors (possibly in a nongenomic role).

Chapter 4

An investigation into the mechanisms by which RA induces electrophysiological changes in cultured identified neurons.

4.01 – Abstract

In addition to its ability to regulate gene expression through the classical retinoid signaling pathway, RA has been implicated (from previous studies) to operate through a myriad of alternative signaling pathways to elicit its effects. Such pathways include (but are not limited to), local protein synthesis, the cyclic AMP pathway, the phospholipase C pathway and calcium signaling. There is also mounting evidence implicating the non-genomic actions of the retinoid receptors in a number of RA-mediated effects. The previous chapter has shown that RA can rapidly elicit changes in the firing properties of neurons. The present chapter will attempt to determine the signaling pathway(s) through which RA operates. The data suggest that protein synthesis, protein kinase A and phospholipase C activation, as well as calcium influx, are not required for RA to elicit its effects. Additionally, this chapter has investigated whether the retinoid receptors play a role in mediating the electrophysiological effects of RA. The data from these experiments suggest that the RXR, but not the RAR, may be involved in the RA-induced changes. Additionally, it is shown that RA exposure results in a significant decrease in intracellular calcium ($[Ca]_i$) that corresponds with the time course, dose- and isomer-dependency of the RA-induced firing changes. Lastly, these studies investigate whether the $[Ca]_i$ decrease results in the alteration of calcium-dependent potassium channels, which are known to play a role in bursting behaviour and spike waveform. These experiments provide evidence to suggest that these channels are not playing a role. These studies provide the first insights into the mechanisms underlying RA's actions on the electrophysiological properties of adult neurons.

4.02 – Introduction

The data from the previous chapter show, for the first time in any species, that RA can cause dramatic changes in the firing properties of adult neurons within minutes of exposure. Application of RA causes the appearance of atypical firing activity, changes in the shape of action potentials and the eventual (transient) silencing of firing activity. Moreover, it was shown that these effects appear to be both dose-dependent and isomer-dependent. Lastly, these studies have shown that the RA-induced changes in firing pattern do not require the provision of trophic support, appear to be independent of the electrical activity of neurons and are not altered by pre-exposure to low concentrations of RA in culture. Having characterized these novel electrophysiological changes, the aim of this chapter was to take the first steps in determining the mechanism by which RA operates to elicit such effects. Classically, RA is well known for its influence on gene transcription, working exclusively through the retinoid receptors, which have been well studied for their transcriptional activation of target genes (Mey and McCaffery, 2004; Theodosiou et al., 2010), particularly during development (Zile, 2001). However, more recent studies have begun to elucidate a number of non-genomic roles the retinoid receptors may have, as well as a number of other "non-classical", retinoid receptor-independent pathways through which RA can operate. In particular, RA has been shown to operate through the activation of various intracellular messenger pathways, such as, protein kinase A (PKA; Kholodenko et al., 2007), phospholipase C (PLC; Liou et al., 2005) and calcium signaling (both calcium influx and release from internal stores; Chen and Napoli, 2008b). These interactions represent possible pathways by which RA may be acting to elicit its changes in the firing properties of neurons.

Recent work has implicated a non-genomic action of the RAR in local protein synthesis (Chen and Napoli, 2008a; Chen et al., 2008). More specifically, RA exposure in mouse hippocampal

neurons can rapidly activate the local translation and expression of target mRNAs in an RAR-dependent manner. This work has shown that the RAR actively binds with various translational regulators and activators to influence local protein synthesis in dendrites. Furthermore, the RAR has been shown to act as a translational repressor, whereby it actively binds to and represses the translation of target mRNAs (Poon and Chen, 2008). This repression is released upon RA-exposure, allowing for the rapid induction of targeted protein synthesis. These combined studies show that the RAR has the capacity to operate outside of the nucleus and, thus, represents a non-genomic action of a retinoid receptor. Additionally, recent work in our lab has provided support for non-genomic actions of the RXR. It has previously been shown that RA can elicit positive growth cone turning of cultured *Lymnaea* neurons in the absence of a cell body (Farrar et al., 2009). Interestingly, this growth cone turning may require the RXR, as growth cones have been shown to turn towards a RXR agonist in the same manner in which they turn towards RA (Carter et al., 2010). Moreover, growth cones have been shown to be unresponsive to RA in the presence of RXR antagonists (Rand et al., 2011). This suggests that the RXR may be operating in a non-genomic capacity to elicit growth cone turning. Taken together, these data show that RA can elicit rapid, non-genomic effects through the retinoid receptors. These studies demonstrate that RA can recruit the retinoid receptors for use in multiple responses, through the classical pathway for transcriptional activation, or through the non-genomic pathway for novel, non-classsical actions.

In addition to operating through the retinoid receptors, recent work has shown that RA can act via a number of second messenger signaling pathways, independently of the retinoid receptors. For example, work utilizing mouse retinal cells has shown that RA-induced increases in cell survivability can be mimicked with agonists of PKA (Kholodenko et al., 2007). This work

suggests that RA may elicit its effects through the activation of PKA, rather than transcriptional activation via the classical retinoid signaling pathway (ie: genes containing a RARE sequence). Additionally, RA has been implicated in modulating the activity of phospholipase C (PLC), a signaling protein involved in various signaling cascades such as those involving calcium release from intracellular stores (Liou et al., 2005). More specifically, RA has been found to elicit increases in spontaneous neurotransmitter release at *Xenopus* neuromuscular junctions in a PLC-dependent manner (Liou et al., 2005). In this work, the RA-induced increases were shown to be abolished in the presence of a PLC inhibitor, suggesting that RA operates through this intracellular messenger pathway. Taken together, these data suggest that RA can elicit changes through non-classical mechanisms involving the activation of the PKA and PLC signaling pathways.

Substantial work has also shown that RA may be able to indirectly elicit changes in calcium signaling in part by modifying the expression of proteins thought to be involved in the calcium signaling cascade. For example, exposure to RA has been shown to cause a dramatic upregulation of calcium/calmodulin protein kinase α (CaMKKα) in mouse hippocampal neurons (Chen and Napoli, 2008b) and during neutrophil maturation (Lawson et al., 1999). CaMKKα is known to activate a wide variety of calcium-dependent signaling cascades, such as cAMP and IP3 activation (Lawson et al., 1999), and thus RAs ability to alter the expression of CaMKKα may modulate a cell's response to calcium signals. Similar work in gastric cancer cells (Shyu et al., 2003) as well as HL-60 cells (Wu et al., 2004) has investigated the expression of SP100 protein, a calcium-binding protein (whose function is currently unknown). Perhaps more directly, RA exposure has been shown to cause modest increases in intracellular calcium concentration ($[Ca]_i$) in both rat and human cultured cell lines, although in both cases the exact mechanism

remains unclear (Short et al., 1991; Gao et al., 1998). Taken together, these data demonstrate that RA may have the capacity to alter calcium signaling within cells, either directly through changes in $[Ca]_i$, or indirectly through modifying the expression of calcium signaling proteins. This is of particular interest to the current studies as calcium signals are thought to play a role in the initiation of neurite outgrowth (McClellan et al., 2008) and to modulate impulse activity (Coulon et al., 2009; Sanchez-Alonso et al., 2010) in neurons. For example, calcium released from intracellular stores has been found to reduce the threshold for the initiation of impulse bursts in neurons (Coulon et al., 2009; Sanchez-Alonso et al., 2010). Taken together these data demonstrate that RA may have the capacity to alter $[Ca]_i$, which may provide a possible mechanism for the effects on cell firing documented in the previous chapter.

Interestingly, the studies described above typically document rapid changes in response to RA exposure, suggesting the possibility of a non-classical mechanism of action. Since the responses characterized in the previous chapter also occur rapidly, often within 15 - 30 minutes after exposure, it is possible that RA is acting in a non-genomic fashion. Additionally, the previous chapter showed RA's ability to change the firing pattern of an isolated neurite, in the absence of a nucleus, which adds support for the notion that RA may be acting through one or more of these non-classical pathways to elicit its effects. As a first step, initial studies were performed in an effort to determine whether RA may be acting through protein synthesis, the cAMP signaling (specifically via PKA), the PLC signaling pathway and through calcium influx through voltage-gated calcium channels. Alternatively, RA may be utilizing the retinoid receptors (or a retinoid receptor) in a non-genomic capacity to induce the changes in firing pattern. To determine whether this was the case, the next aim of this chapter was to determine if the RA-mediated effects on firing pattern could be mimicked by RAR or RXR agonists, or impaired by RAR or

RXR antagonists. Lastly, RA may be exerting its effects on the firing activity of neurons by altering $[Ca]_i$ levels. Thus, the final aim of this chapter sought to determine if RA exposure results in changes to $[Ca]_i$ levels. Taken together, this chapter will comprise a series of investigations to address a wide variety of potential mechanisms or pathways through which RA may operate to elicit its electrophysiological effects. These studies represent the first steps ever taken to determine how RA elicits its novel effects on cell firing.

4.03 - Materials and Methods

Cell Culture & Electrophysiology. All procedures and equipment used were identical to those described previously in Chapter 3.

Chemicals. All chemicals were purchased from Sigma-Aldrich unless otherwise stated. *All-trans* and 9-*cis* retinoic acid stocks were made fresh daily and dissolved in DM. In some experiments neurons were incubated in the presence of antagonists or inhibitors prior to the start of electrophysiological recording. Unless otherwise stated, solutions with antagonists or inhibitors were made fresh daily from 10mM aliquot stock solutions dissolved in deionized water. The RAR agonist TTNPB was obtained from Tocris Bioscience. The RXR agonist, PA024, the RXR antagonists HX531 and PA452 as well as the RAR antagonist LE540 were all obtained from Dr. Kagechika (University of Tokyo, Japan). In all cases, the agonist and/or antagonist was used at a final bath concentration of either 10μM or 1μM and were applied to the bath via a micropipette. Vehicle control solutions for either agonist or antagonist experiments was 0.1% DMSO dissolved in DM. Anisomycin, used to block protein synthesis, was added to the bath for a final concentration of 45μM. To block calcium influx, a bath concentration of 10μM or 30μM cadmium was used. The PKA inhibitor, Rp-Adenosine 3'5'cyclic monophosphorothioate (Rp-cAMPs) and phospholipase C inhibitor, U-73122, were used at final bath concentrations of 10μM and 20μM, respectively. Vehicle control solutions for the above conditions were 0.1% EtOH dissolved in DM (for work utilizing second messenger pathway experiments) or 0.1% DMSO (for retinoid receptor agonist/antagonist experiments). All antagonists and inhibitors were added to the bath at least one hour prior to the start of recording. Indo-1 AM ester was obtained from Invitrogen. Working solutions of Indo-1 AM were made fresh daily from frozen aliquots of 1mM stock solution dissolved in 100% anhydrous DMSO.

Spike Waveform Analysis. All procedures and equipment used were identical to those described previously in Chapter 2.

Neuronal loading of Indo-1 AM ester into neurons. One day after plating cells onto culture dishes the calcium indicator dye indo-1 AM was loaded into neurons. Briefly, the culture medium was replaced with saline (via a perfusion pump), after which the indo-1 AM ester was added to the dish using a pipette to give a final bath concentration of 1 μM. Cells were then incubated at 21°C for 3 hours. The AM ester allows the dye to be taken up by cells. Once in the cytoplasm, the AM group is cleaved by endogenous esterases, producing a functional indo-1 dye which is membrane impermeable. After dye loading, cells were washed with saline to remove unloaded indo-1 AM dye from the dish.

Fluorescence imaging of neurons with indo-1 loaded neurons. Once loaded, neurons were imaged on an inverted microscope (Olympus Inc., Ontario, Canada) with two photon fluorescence capabilities (special thanks to Dr. Doug Bruce, Department of Biological Sciences, Brock University). More specifically, a Tsunami mode locked Ti: Sapphire laser (model 3960) was set to a 740nm excitation wavelength. Two bandpass filters were used to detect both the calcium-free and calcium-bound indo-1 dye (detection wavelengths of 480±25nm and 405±25nm, respectively). Fluorescent emission from both wavelengths was simultaneously detected from an Olympus Fluoview FV300 scan unit, and Fluoview software (v 5.0) was used to acquire images on a computer. Time-lapse fluorescence images of cultured neurons were taken at 5 minute intervals, for 20 minutes prior to and one hour after application of RA. These experiments were performed in collaboration with Dr. Ye Yuan and Dr. Doug Bruce (Brock University).

Imaging analysis. Images were processed with ImageJ software (v 1.42q) utilizing a custom macro written to calculate the fluorescence from a time-lapse series of two channel images which have been background-subtracted (designed by Dr. Ye Yuan). For each image in a time-lapse series the perimeter of the imaged neuron was hand traced, and the ratio of the fluorescence within the neuron was measured at 405nm (representing bound dye) and 480nm (representing free dye) was calculated. The ratio of free to bound dye during a 20 minute period immediately prior to RA exposure was averaged and taken as the pre-exposure (baseline) level. Changes in intracellular calcium during the 60 minutes of RA exposure procedure are expressed as a percent change from this value.

Statistical Analysis. Analysis of data was performed with SigmaStat v3.5 software (SigmaStat Software Inc., Virgina, USA). Differences between groups at a specific time point were determined using a one- way ANOVA on data normalized to pre-exposure values (set at 0%). If significant differences were found ($p < 0.05$), a Tukey-Kramer *post hoc* test was performed. Any data set that did not meet the assumptions of the ANOVA was either log or reciprocally transformed prior to further analysis. In the event that data did not meet normality conditions, a one way Kruskal-Wallis was performed followed by a Dunn's *post hoc* test. The presence or absence of plateau potentials and/or atypical impulse activity for each cell was analyzed using Fisher's Exact tests which were then corrected according to the Bonferroni-Holm method. Trials comparing RA with vehicle control, 10μM cadmium, PLC or PKA inhibition were run together and as such, statistical analysis (one way ANOVA) was performed on all groups together. However, for the sake of clarity, some of these data are presented in separate graphs in the results section. All values are expressed as mean ± standard error of the mean (S.E.M.), unless otherwise stated.

4.04 – Results

I. RA-induced firing changes may occur independently of protein synthesis, PKA & PLC activation and calcium influx.

As a first step towards elucidating the underlying cellular mechanisms involved in the RA-induced electrophysiological changes I sought to investigate whether RA's effects were mediated via the activity of various second messenger pathways. In order to determine if protein synthesis is involved I utilized the protein synthesis inhibitor anisomycin. To determine if the PKA or PLC activation is involved I exposed neurons to either the PKA inhibitor Rp-Adenosine 3'5'cyclic monophosphoraite or the phospholipase C inhibitor U-73122, respectively. For simplicity these agents are referred to in figures as the PKA inhibitor (PKAi) and PLC inhibitor (PLCi), respectively. Lastly, to determine if calcium influx (considered here as a messenger pathway since changes in calcium influx can also lead to widespread physiological effects) plays a role in the RA-induced firing changes, I utilized the voltage-gated calcium channel blocker, cadmium.

In the following experiments, each antagonist or inhibitor was applied at least one hour prior to impaling neurons and the start of recordings. After one hour all neurons were then exposed to 10μM atRA and recordings were analyzed in a fashion identical to that performed in the previous chapter. More specifically, recordings were analyzed for the presence of atypical firing activity as well as changes in spike waveform (half-amplitude duration, rise time and decay time). Additionally, the RMP, input resistance as well as frequency-dependent broadening were measured.

None of the antagonists or inhibitors were able to prevent or impair the RA-induced increases in atypical impulse activity. In all cases, neurons exposed to RA, regardless of pre-exposure to antagonist or inhibitor, showed high levels of atypical impulse activity (Figure 4.1: A, 40-70% depending upon specific condition compared to 5% in EtOH controls). As shown in Figure 4.1 A, neurons pre-exposed to antagonist or inhibitor and then acutely exposed to RA did not show a significantly different prevalence of atypical impulse activity when compared to neurons exposed to RA alone (n=28). These data suggest that the prevalence of atypical impulse activity does not appear to be impaired in cells with inhibited protein synthesis (n=14), PKAi (n=18) or PLCi (n=17) or blockade of calcium influx with cadmium (n=19), suggesting that these pathways may not be required to elicit RA's electrophysiological effects.

When investigating RA-induced cell silencing, it was found that virtually all (96%, 24/25) EtOH -exposed neurons were still able to fire spontaneous or evoked action potentials one hour after exposure, whereas only 7% (2/28) of RA-exposed neurons were able to do so, as seen previously. For these neurons pre-exposed to inhibitors, approximately 22% (4/18), 24% (4/17) and 6% (1/17) of neurons exposed to anysomycin, PKAi or PLCi were able to fire action potentials after 60 minutes of RA exposure, respectively. Lastly, only 16% (3/19) of neurons pre-exposed to cadmium were able to fire action potentials after 60 minutes of RA exposure. In all cases, neurons pre-exposed to antagonist or inhibitor and then exposed to RA were found to be significantly different when compared to EtOH controls, but not from neurons exposed to RA alone (Figure 4.1). These data suggest that, like the results obtained for atypical impulse activity, RA's ability to induce cell silencing does not appear to require protein synthesis, PKA or PLC activation or calcium influx.

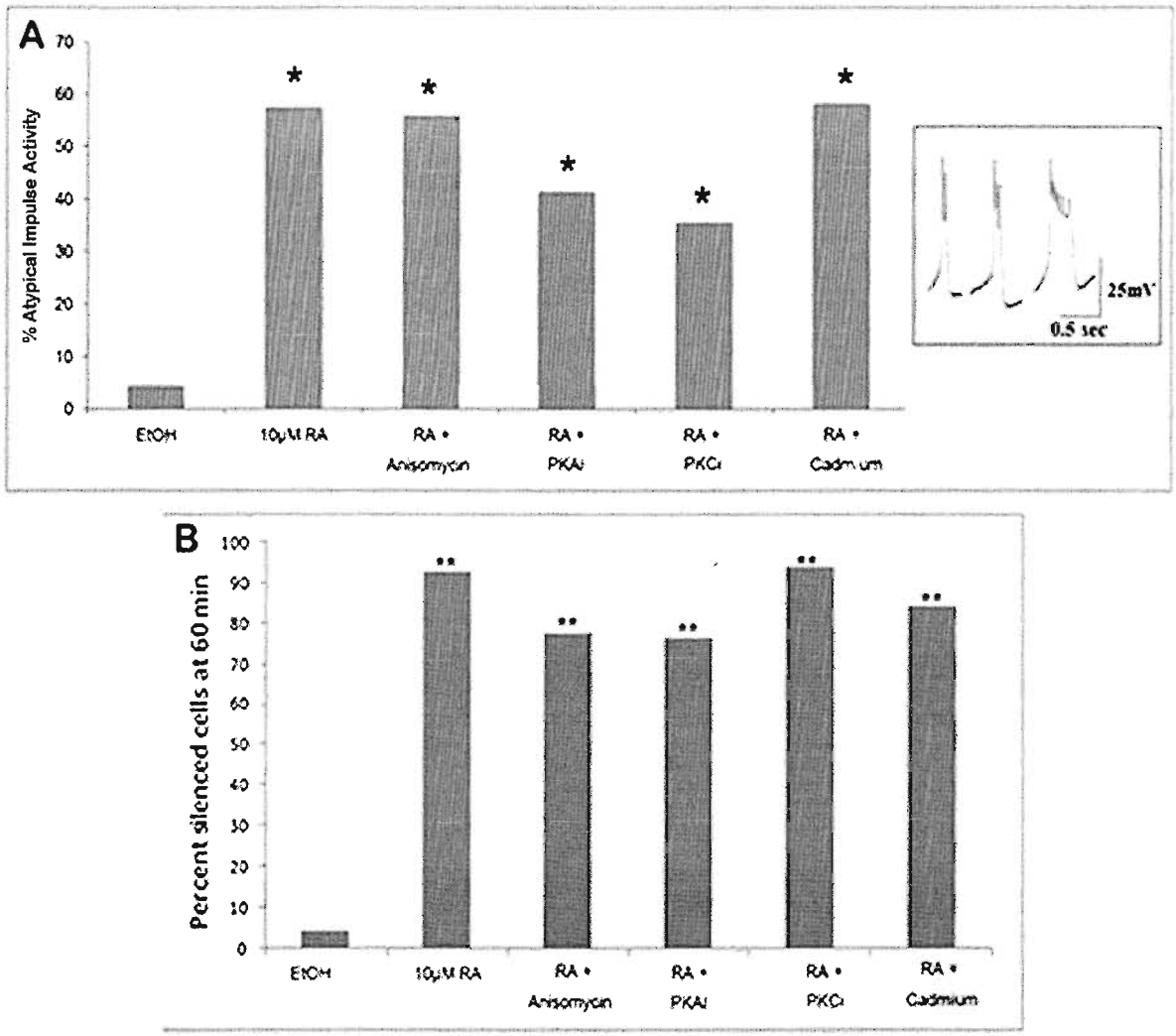


Figure 4.1. Presence of atypical impulse activity and RA-induced cell silencing is not significantly changed in the presence of anisomycin, PKA or PLC inhibitors and cadmium. A: The probability of displaying atypical impulse activity in neurons pre-exposed to each inhibitor and RA, was not significantly different from neurons exposed to RA alone. All conditions were significantly different from control (EtOH). B: The number of silenced neurons pre-exposed to inhibitor and RA was not significantly different from neurons exposed to RA alone. All conditions were found to be significantly different from control. (*p <0.05, **p<0.01).

Analysis of spike waveform, specifically the half-amplitude duration and decay time at 15 and 35 minutes of RA exposure, revealed that the protein synthesis inhibitor did not alter the RA-induced increases in acutely exposed neurons (when compared to EtOH controls; Figure 4.2, A & B). Similarly, the PKA or PLC inhibitors did not appear to alter the RA induced increases (Figure 4.2, A & B; white vs. light grey and striped bars), suggesting that, like protein synthesis, these signaling pathways might not be involved in the RA-induced increases in half-amplitude duration and decay time. Lastly, calcium channel blockade with cadmium failed to inhibit the RA-induced firing changes completely (Figure 4.2, A & B, white vs. brick bar). Interestingly, while significantly different from controls, cadmium-exposed neurons also showed a significant difference in the half-amplitude duration when compared to neurons exposed to RA alone after 35 minutes of RA exposure (Figure 4.2, black vs. brick bar). These data suggest that cadmium may be eliciting a partial inhibition of the RA-induced effect. In all other conditions and time points no significant differences were found when compared to RA treatment alone. These data suggest that, similar to atypical impulse activity, protein synthesis as well as PKA and PLC signaling may not be required to elicit the RA-induced increases in half-amplitude duration and decay time. The role of calcium influx through voltage-gated calcium channels, however, requires further investigation, as the data suggest that calcium influx may play a role in some of the RA-induced electrophysiological changes.

Lastly, when comparing frequency-dependent impulse broadening, no difference was found between the mean train frequency of neurons pre-exposed to antagonist or inhibitor and RA and neurons exposed to RA alone. As shown in Figure 4.3, the frequency-depending broadening between neurons pre-exposed to anisomycin (n=11), PKA inhibitor (n=12) or PLC inhibitor (n=9) and then acutely exposed to RA were all found to be significantly different from EtOH

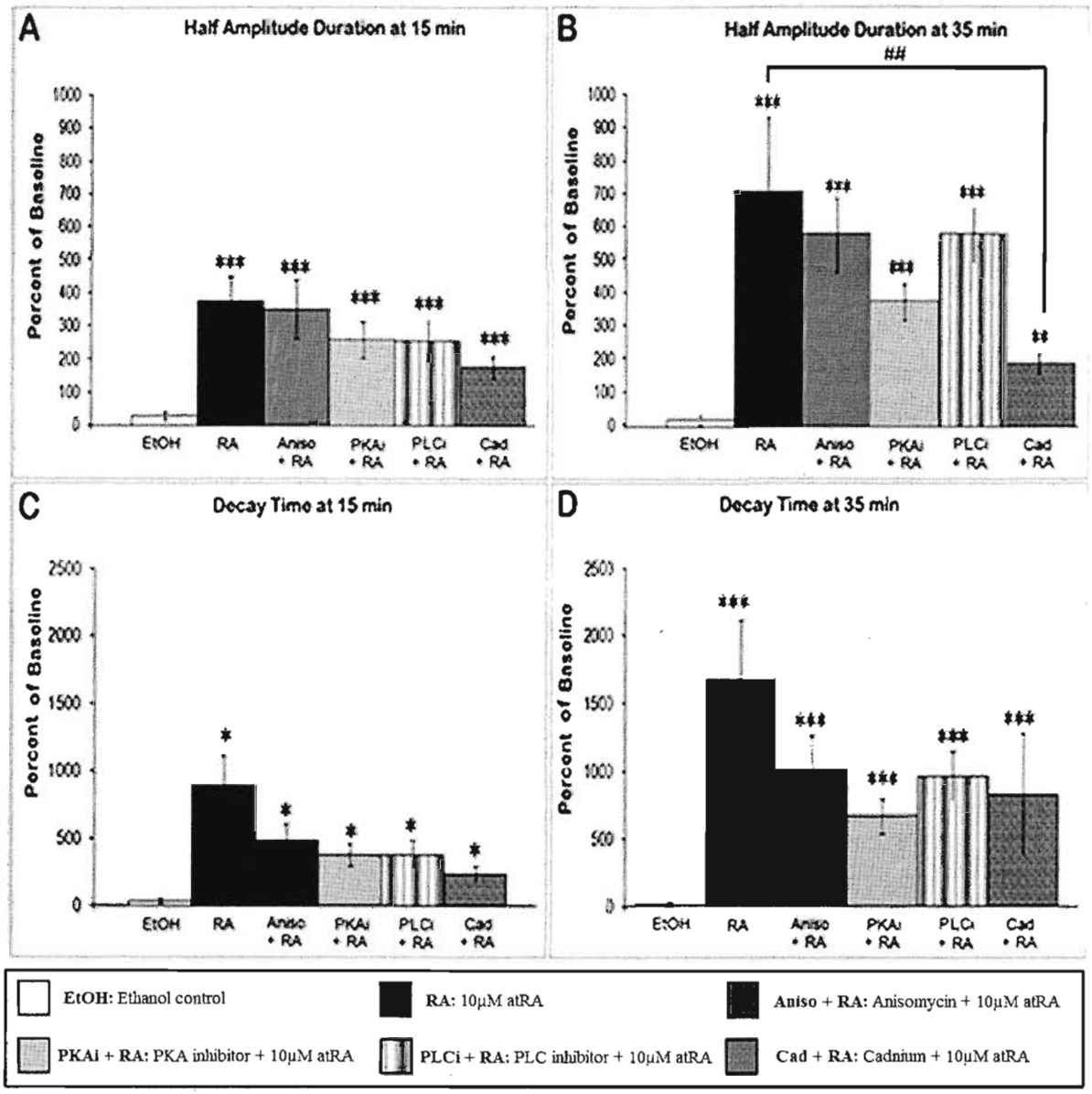


Figure 4.2. Half-amplitude duration and decay time are unaffected by inhibitors of protein synthesis, PKA & PLC and calcium influx. Spike analysis of the half-amplitude duration 15 (A) and 35 (B) minutes after application of RA in the presence of anisomycin, PKA or PLC inhibitors, and cadmium, taken as a percent of baseline (set at 0%). All conditions were found to be significantly different from EtOH controls, but not significantly different from RA exposure alone (with the exception of the cadmium condition after 35 minutes after RA exposure). Decay time of neurons 15 (A) and 35 (B) minutes after application of RA. All conditions were found to be significantly different from EtOH controls, but not significantly different from RA applicatoin alone. In all cases, the RA-induced increases were more dramatic at the 35 minute time point. Replicates for 15 minute analysis: anisomycin (n=14), PKAi (n=17), PLCi (n=12), cadmium (n=17), atRA (n=22), EtOH (n=22). Replicates for 35 minute analysis: anisomycin (n=8), PKAi (n=7), PLCi (n=4), cadmium (n=8), atRA (n=6), EtOH (n=8). (* = $p<0.05$, ** = $p<0.01$ and ***=$p<0.001$, when compared to control; ##=$p<0.01$ when compared to RA).

Table 5 – Peak to peak amplitude, rise time and input resistance are unaffected by inhibitors of protein synthesis, PKA & PLC and calcium influx. Action potential waveform analysis at 35 minutes of exposure to RA alone or co-exposure of RA and antagonist/inhibitor. Input resistance was measured 10 minutes prior to and one hour after RA exposure. All values are expressed as a percent of baseline (set at 100%). In all cases no differences were found.

(% of baseline)	**10 µM RA**	**Anisomycin + RA**	**PKAi + RA**	**PKCi + RA**	**Cadmium + RA**
Peak to Peak Amplitude (%)	89.6 ± 2.7	93.4 ± 4.8	94.0 ± 2.8	91.1 ± 5.1	93.7 ± 3.3
Rise Time (%)	188.8 ± 40.9	155.9 ± 38.0	130.1 ± 11.7	129.8 ± 27.8	112.3 ± 22.4
Input Resistance (%)	113.1 ± 13.1	98.7 ± 10.7	84.1 ± 14.1	74.4 ± 11.2	123.1 ± 22.3

controls (n=17) but not from neurons exposed to RA alone (n=17). Interestingly, impulse broadening in neurons pre-exposed to cadmium and RA (n=10) were not significantly different from controls or from neurons exposed to RA alone. Taken together, these data suggest that RA-induced effects on impulse broadening may be independent from protein synthesis, as well as PKA and PLC signaling. Similarly to the spike waveform analysis, these data suggest that calcium influx may play a role, as blockade of these channels reduced the RA-induced effects.

The above data indicate that a possible role of calcium influx in the RA-induced changes requires further evaluation. My initial experiments utilized a 10μM cadmium concentration, which our lab has previously used to block RA-induced growth cone turning responses (Farrar et al., 2009). Given that concentrations as high as 50μM have been used in *Lymnaea* (Molnar et al., 2004), I hypothesized that the concentration used in my initial experiments may have produced only a partial blockade of voltage-gated calcium channels, which may have resulted in only a partial block of the RA-induced changes. Therefore, I repeated the previous experiments utilizing a higher concentration of 30μM cadmium. In these experiments a new group of neurons exposed to RA alone was tested in parallel and used as a positive control.

Overall, there was no significant difference in the percentage of neurons showing atypical impulse activity in neurons treated with 30μM cadmium and RA (4/12) compared to neurons treated with RA alone (7/13) (Figure 4.4, A). Additionally, no significant difference was found in RA-induced cell silencing between either group (Fisher's exact test performed; RA: 13/16 cells silenced, 30μM cadmium + RA: 8/12 cells silenced). These data indicate that exposure to a higher dose of cadmium does not alter RA's ability to induce atypical impulse activity or elicit cell silencing. When analyzing both the half-amplitude duration as well as decay time, no

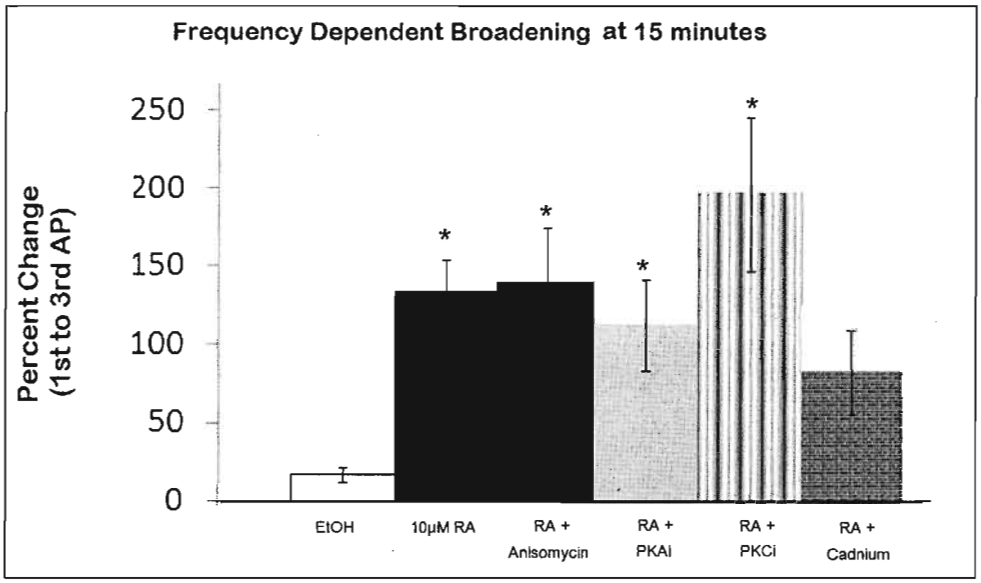


Figure 4.3. Anisomycin, PKAi and PLCi do not block RA-induced increases in frequency-dependent broadening. Frequency-dependent broadening of neurons after 15 minutes of exposure 10μM RA in the presence of anisomycin, PKAi, PLCi, cadmium or RA alone (first action potential set at 0%). Neurons exposed to 10μM RA, regardless of co-exposure to antagonist or inhibitor, were found to be different from EtOH controls. Neurons co-exposed to cadmium and 10μM RA were not found to be significantly different from controls or neurons exposed to RA alone. (* = $p<0.05$)

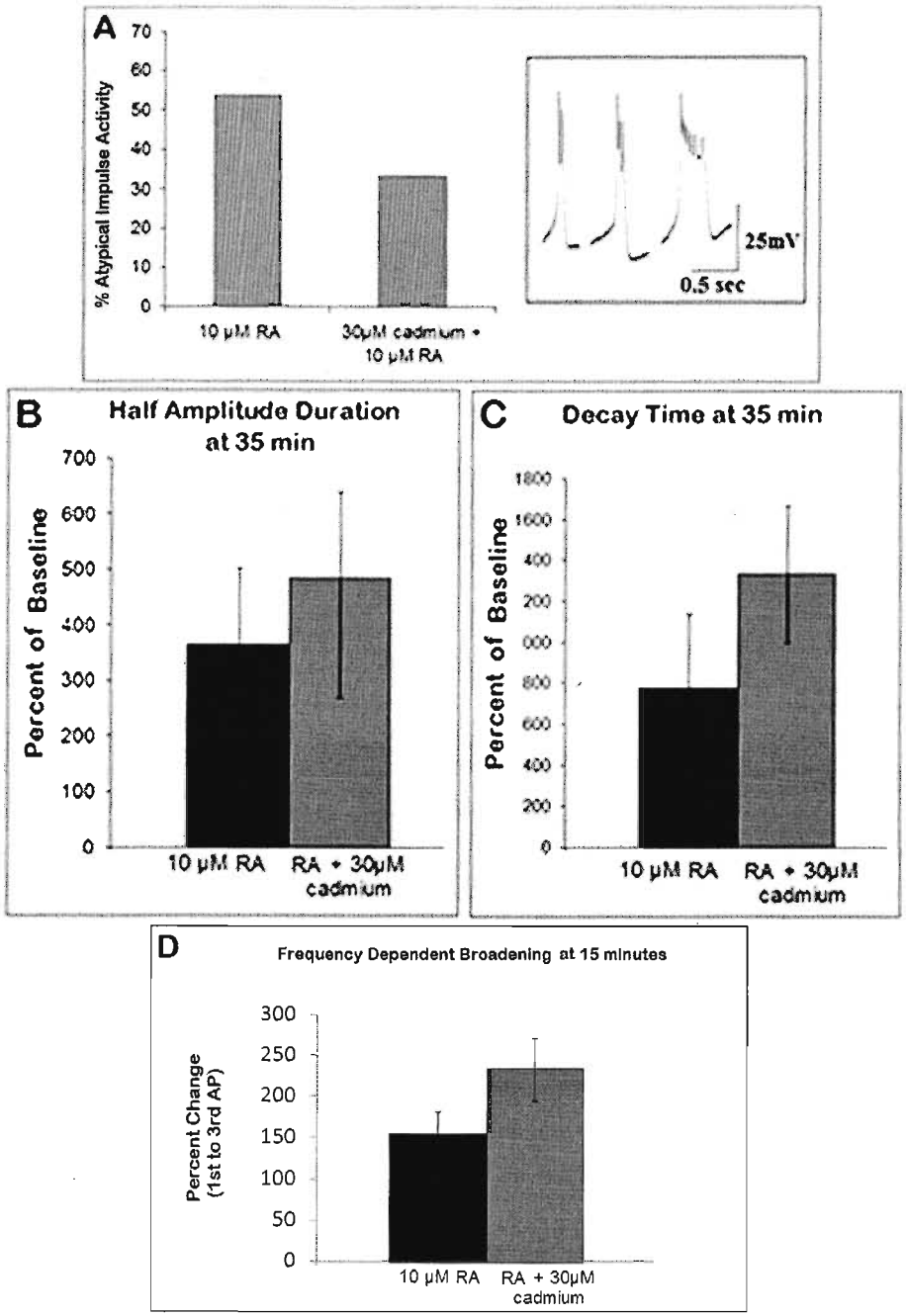


Figure 4.4. RA-induced changes in impulse activity and spike waveform are independent of calcium influx through voltage gated calcium channels. No significant difference in the percent of neurons displaying atypical impulse activity (A) between neurons pre-exposed to 30µM cadmium and then acutely exposed to RA compared to neurons exposed to RA alone. The RA-induced increase in half-amplitude duration (B) and decay time (C) 35 minutes after retinoid exposure was not significantly different between neurons exposed to 30µM cadmium and RA or RA alone (baseline set at 0%). No difference was found in the frequency dependent broadening (D) of neurons in either treatment group (shown as the percent of the first action potential, set at 0%).

significant difference in neurons treated with 30μM cadmium and RA or RA treatment alone was found at any time point (30μM cadmium + RA: n=11, RA alone: n=13; Figure 4.4, B & C). Similarly, no significant differences were found in the rise time and peak to peak amplitude of action potentials at any time point (data not shown). Furthermore, no significant difference was found in the input resistance (30μM cadmium + atRA: n=6, atRA alone: n=13) or RMP (30μM cadmium + atRA: n=9, atRA alone: n=8) of either group, 60 minutes after retinoid exposure. Lastly, no significant differences were found in frequency-dependent impulse broadening when comparing neurons pre-exposed to 30μM cadmium and RA (n=7) to neurons treated with RA alone (n=11; Figure 4.4, D). Thus, these data suggest that the higher dose of cadmium did not appear to block the RA-induced electrophysiological changes. Therefore, these data strongly suggest that calcium influx through voltage-gated calcium channels does not appear to play a role in the RA-induced firing changes.

II. RA-induced effects may be dependent on RXR signaling, but not RAR signaling.

The previous experiments suggested that RA may be eliciting its effects through some means not requiring protein synthesis, PKA or PLC activation, or calcium influx through voltage-gated calcium channels. Thus, I next sought to determine if the RA-induced changes in firing properties operate through the retinoid receptors. Both the RAR and RXR have been implicated in a number of non-genomic actions (Chen and Napoli, 2008a; Chen et al., 2008; Zhelyaznik and Mey, 2006), and our lab has shown the cytoplasmic localization of the RXR (Carter et al., 2010) as well as the RAR (Carter, 2011) in the neurites and growth cones of cultured neurons. Importantly, recent work in our lab has shown that a RXR agonist can elicit growth cone turning in neurites devoid of a cell body (Carter et al., 2010). These data suggest that RA has the capacity to elicit effects through non-genomic actions of the retinoid receptors and, thus, warrant further investigation to determine if such a pathway is utilized in the electrophysiological

responses to RA. To this end I conducted two series of studies utilizing a pharmacological approach. First, I utilized RAR and RXR selective agonists (TTNPB and PA024, respectively) which have previously been used in *Lymnaea* (Carter, 2011; Farrar et al., 2009) and examined whether either agonist alone could mimic the electrophysiological changes exerted by 10μM RA. Second, I sought to determine if targeted inhibition of either the RAR or RXR could block the RA-induced changes in firing pattern. To this end I utilized a RAR pan-antagonist, LE540, and two RXR pan-antagonists, HX531 and PA452, which have also been used in previous *Lymnaea* studies (Farrar et al., 2009) as well as a number of vertebrate systems (Bianchi et al., 2009; Del Rincon and Scadding, 2002; Konta et al., 2001; Kagechika, 2002;).

i. RAR and RXR agonists do not appear to induce changes in firing pattern.

In order to determine whether the RAR or RXR agonists could induce changes in firing pattern as seen with RA-exposed neurons, I applied either the RAR agonist TTNPB (10μM) or the RXR agonist PA024 (10μM) to the bath and monitored both firing activity and spike waveform for 60 minutes after application. Control experiments consisted of application of 0.1% dimethyl sulfoxide (DMSO), the vehicle in which these agents were dissolved. A positive control in these experiments consisted of a new group of 10μM RA-exposed neurons.

Preliminary experiments utilizing 10μM TTNPB showed that only 20% (2/10) of neurons were able to fire spontaneous or evoked action potentials 35 minutes after exposure. Furthermore, all cells became silent within 50 minutes of application. While these initial results may suggest that 10μM TTNPB was able to mimic RA's cell silencing effects, a more detailed analysis suggested that cell viability was compromised by this concentration of the agonist. More specifically, I

have shown that application of RA results in transient cell silencing, which is restored 3 hours after exposure. In a separate series of experiments neurons were exposed to a three hour incubation in 10μM TTNPB, and recordings were made subsequently. Nearly all neurons (19/22) had no measurable RMP (and displayed no action potential firing). In this condition, all DMSO-exposed cells (17/17) remained electrically excitable 3 hours after incubation, indicating that at this concentration TTNPB adversely affects the cell's ability to maintain a RMP. Further studies with 5μM TTNPB produced similar results, with no cells being suitable for recording after application (n=18). Thus, examination of any firing pattern changes was not possible at either the 10μM or 5μM TTNPB concentrations, so I utilized a lower (1μM) concentration for further studies. At this concentration virtually all cells (11/12) were able to fire impulses up to one hour after TTNPB application.

As shown in Figure 4.5, A, neurons exposed to either the RAR agonist TTNPB (1μM) and RXR agonist PA024 (10μM) did not show a high prevalence of atypical impulse activity. No neurons exposed to TTNPB (0/12) and only 20% (2/10) of neurons exposed to PA024 showed atypical impulse activity, whereas 71% (12/17) of neurons exposed to RA displayed atypical impulse activity. Significantly more RA-exposed neurons were found to have atypical impulse activity compared to either agonist condition or EtOH controls (n=12). Similar results were observed when RA-induced cell silencing was analyzed (Figure 5, B). These data suggest that the actions of the retinoid receptors may not be required to elicit these specific RA-induced firing changes.

I next compared the spike waveform 15 and 35 minutes after application of RA or the RA agonist. Neurons exposed to RA (n=14) had significantly increased half-amplitude durations and decay times compared to TTNBP (n=11) and PA024 (n=12) exposed neurons 15 minutes after

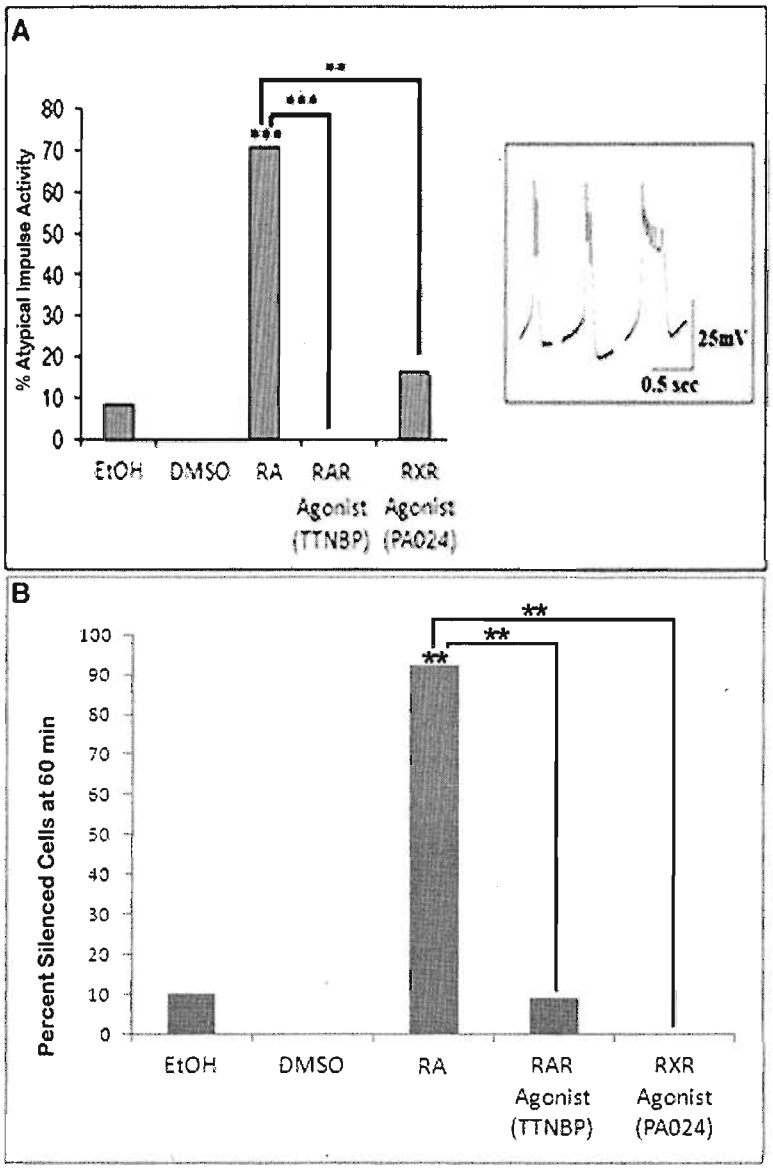


Figure 4.5. RAR and RXR agonists TTNPB and PA024 do not elicit atypical impulse activity or cell silencing in RA-exposed neurons. A: Significantly more neurons exposed to 10μM RA showed atypical impulse activity compared to 1μM TTNPB, 10μM PA024 and EtOH (control) groups. Neither agonist was found to elicit significantly more atypical impulse activitywhen compared to DMSO (control). B: Significantly more neurons exposed to 10μM RA showed cell silencing compared to TTNPB-, PA024- and EtOH-exposed neurons. Neither agonist was found to elicit significantly more cell silencing when compared to DMSO control. An asterix above a bar represents a difference from control conditions (** = $p<0.01$ and ***=$p<0.001$).

application (Figure 4.6, A & C). Similar results were obtained when examining the half-amplitude duration and decay time at 35 minutes post application; however, the effect of RA exposure was more dramatic (Figure 4.6, B & D). Interestingly, the half-amplitude duration in both TTNBP- and PA024-exposed neurons increased slightly by the 35 minute time point, leading to a significant difference compared to DMSO controls (n=13), but this increase was significantly less than that induced by RA, as the half-amplitude duration of RA-exposed neurons (~850%) was more than 8 times the increase induced by either TTNBP (~ 50%) or PA024 (~100%). Lastly, no change was found in either the peak to peak amplitude or rise time of neurons at any time point, and no change was found in the input resistance or the RMP of neurons in all conditions (Appendix 1.05). These results indicate that application of the RAR agonist TTNPB or RXR agonist PA024 may elicit RA-like increases in the half amplitude duration or decay time of action potentials, however these changes are significantly reduced compared to that of neurons exposed to RA. Thus, it is unclear whether these receptors are required to elicit the RA-induced effects.

Lastly, as with my previous experiments, I next examined the frequency-dependent broadening of action potentials within impulse trains. In line with previous results, frequency-dependent impulse broadening of neurons exposed to RA alone (n=13) displayed a significant increase compared to EtOH controls (n=9). Both the TTNBP (n=11) and PA024 (n=10) agonist-exposed groups did not display a similar increase; each was significantly different compared to RA treatment alone (Figure 4.6, E), and neither condition was significantly different from either EtOH or DMSO controls. In agreement with the previous analyses, these data do not indicate that either agonist can mimic the RA-induced changes in firing pattern.

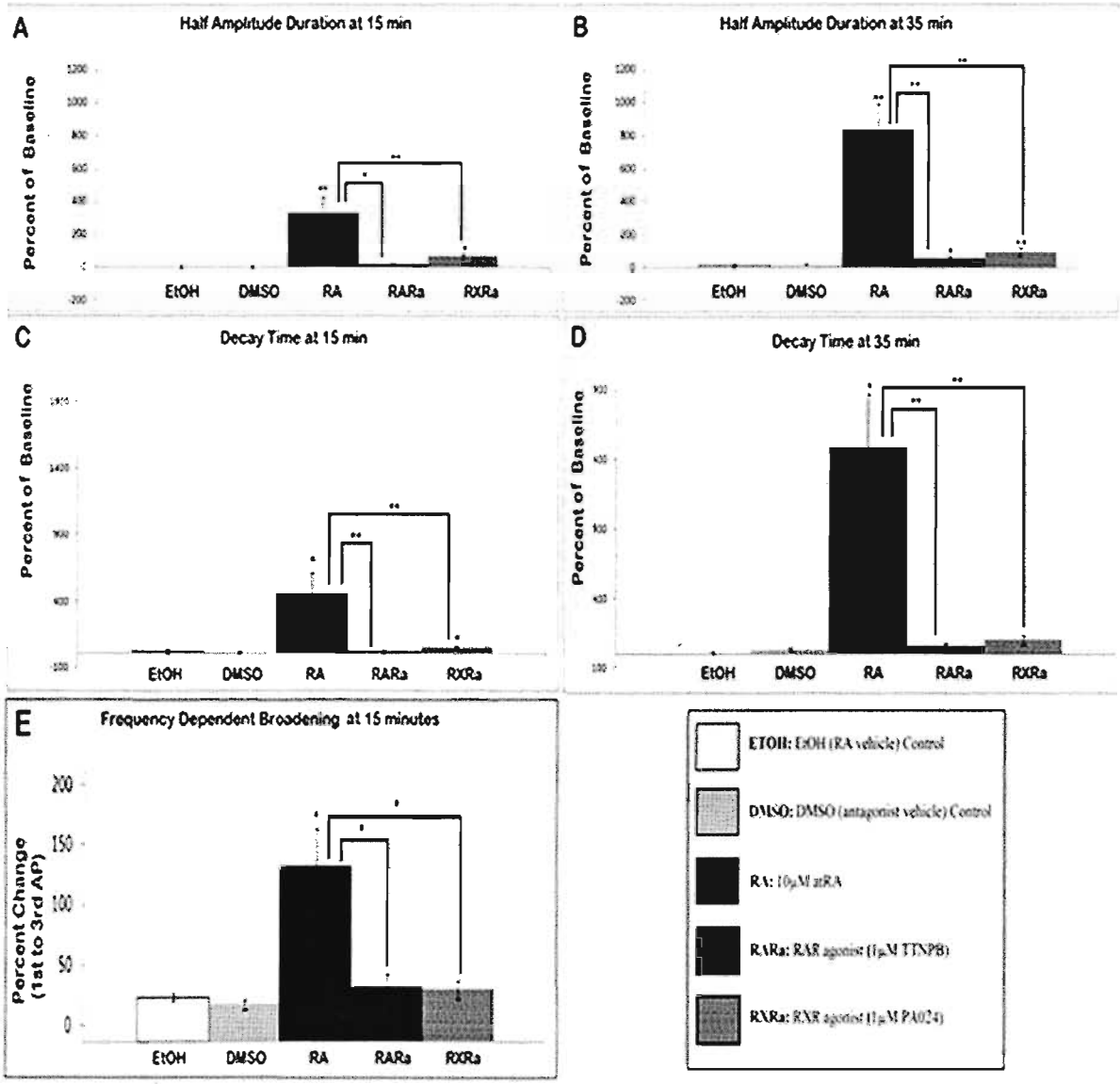


Figure 4.6. RAR and RXR agonists do not induce RA-like changes spike waveform or frequency-dependent broadening. Neurons exposed to RA exhibited significantly increased half-amplitude duration compared to RAR or RXR agonist-exposed neurons and to EtOH controls at both 15 (A) and 35 (B) minutes of exposure. RXR agonist-exposed neurons were significantly different from DMSO controls at both time points. The decay time of neurons exposed to RA was significantly increased compared to the RAR or RXR agonist-exposed neurons as well as EtOH controls both 15 (C) and 35 (D) minutes of exposure. E: Neurons exposed to 10μM RA, but not RAR agonist or RXR agonist, displayed significantly increased frequency-dependent broadening when compared to EtOH controls. RA-exposed neurons had significantly increased frequency-dependent broadening compared to either RAR or RXR agonist-exposed neurons. Data from both agonist trials were not significantly different from those of DMSO vehicle control trials. (baseline set at 0%; Replicates for 15 minute data: 10μM RA: n=14, 1μM TTNPT: n=12, 10μM PA024: n=12, EtOH: n=12, DMSO: n=13. Replicates for 35 minute data: 10μM RA: n=9, 1μM TTNPT: n=11, 10μM PA024: n=12, EtOH: n=11, DMSO: n=13; *=$p<0.05$, **=$p<0.01$).

In summary, these data provide the first evidence suggesting that RAR and RXR agonists (at the concentrations used) were unable to mimic the RA-induced changes in impulse firing pattern. Thus the next approach was to determine whether the retinoid receptor antagonists could block the RA-induced effects.

ii. Blockade of the RXR, but not RAR, impairs RA-induced changes in firing pattern.

The second approach to determine the potential involvement of the RAR or RXR in the RA-induced changes in firing pattern was to determine whether RAR or RXR antagonists could block the RA-induced changes. To this end I utilized the RAR pan-antagonist, LE540, and two RXR pan-antagonists, HX531 and PA452. To ensure adequate impairment of the targeted receptor, each antagonist was applied to the bath at least one hour prior to the start of recording, as performed in our lab previously (Farrar et al., 2009), after which neurons were impaled for electrical recording and exposed to10μM RA. The control condition in these experiments was to incubate neurons for one hour in the presence of 10μM DMSO (vehicle for the antagonists), followed by application of 10μM EtOH (vehicle for RA).

Preliminary experiments utilizing 10μM LE540 (n=11) and 10μM HX531 (n=10) revealed that immediately after electrode insertion, and prior to RA addition, neurons became silent and were unable to fire spontaneous or evoked action potentials. Therefore, the concentration of both antagonists was reduced tenfold to 1μM. At this concentration 57% (10/14) of LE540-treated and 32% (6/19) of HX531- treated neurons were able to fire impulses 35 minutes after 10μM RA application. Although neurons exposed to 10μM PA452 were initially able to fire action potentials, nearly all neurons (17/19) were found to go silent within 35 minutes after application of RA. Thus, no comparison can be made with this antagonist at 35 minutes post-application.

As shown in Figure 4.7 A, RA exposure (n=17) caused a significant increase in atypical impulse activity when compared to the vehicle control in EtOH (n=13). Neurons incubated in 1μM RAR antagonist LE540 (n=14), while decreased in prevalence, were not found to be different from neurons exposed to RA alone. At 1μM, the RXR antagonist, HX531 (n=19) appeared to abolish the RA-induced atypical impulse activity when compared to RA-exposed neurons. Interestingly, at 10μM, the RXR antagonist PA452 (n=18) showed no blockade of RA-induced impulse activity. These neurons were found to display significantly more atypical firing activity when compared to controls. Taken together, these data provide further support that the RAR might not be involved in the RA-induced firing changes. The role of the RXR, however, remains somewhat unclear as different antagonists provided different results.

When examining RA-induced cell silencing it was found that, as expected, only 9% (1/11) of neurons incubated in DMSO and then exposed to EtOH for 60 minutes were unable to fire impulses, suggesting that the vehicle solution does not alter cell firing. Additionally, as expected, 92% (12/13) of neurons exposed to RA for 60 minutes were unable to fire action potentials. Pre-exposure to LE540 or HX531 for one hour, followed by 60 minutes of RA-exposure was found to elicit similar responses, with 75% (9/12) and 95% (18/19) of cells being unable to generate spontaneous or evoked action potentials by the same time point (Figure 4.7, B). The percentage of silent neurons was not significantly different between neurons pre-exposed to either antagonist and neurons exposed to RA alone, but it was significantly different from controls. These data suggest that blockade of the RAR or RXR did not impair the RA-induced cell silencing effects.

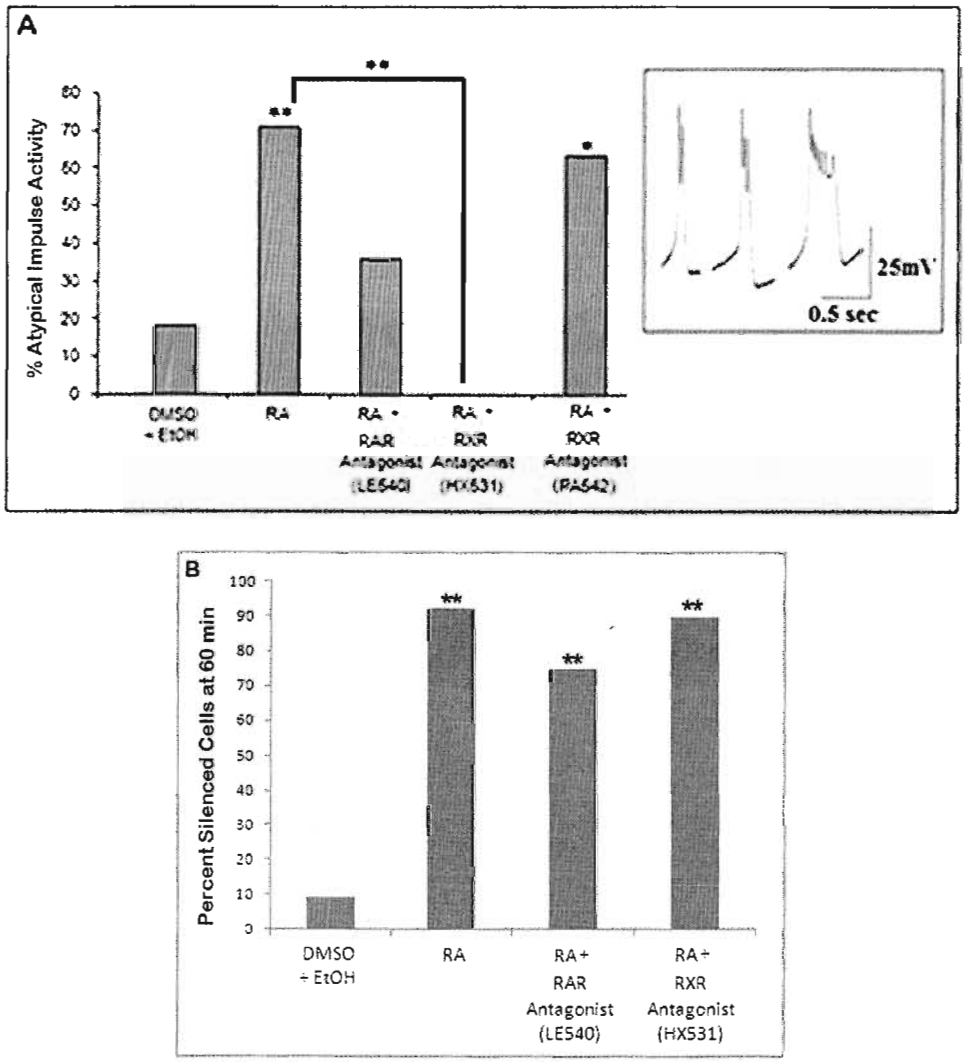


Figure 4.7. The RXR inhibitor HX531 blocks RA-induced atypical impulse activity, but not RA-induced cell silencing. A: Significantly more neurons incubated in RA alone for 60 minutes showed atypical impulse activity than neurons incubated in HX531 which then received RA exposure. The RA-treated groups were significantly different from the controls. The PA452-treated group, while significantly different from control was not significantly different from the RA-treated group alone. No difference was found between the LE540 and control groups. B: Neurons incubated in RA alone showed significantly more cell silencing 60 minutes after exposure. Neurons incubated in LE540 and HX531 for one hour and then exposed to RA were significantly different from controls, but were not different from neurons exposed to RA alone. (*=$p<0.05$, ** = $p<0.01$).

I next compared the spike waveform 15 and 35 minutes after application of RA. Neurons incubated in LE540 or PA452 for one hour, followed by RA exposure, while significantly different from controls, showed no significant difference from neurons exposed to RA alone (Figure 4.8, A-D). Interestingly, neurons exposed to the RXR antagonist, HX531, displayed a significantly smaller increase in half-amplitude duration and decay time at both 15 and 35 minutes after application of RA. Similarly, the increases in frequency-dependent impulse broadening were significantly smaller in neurons treated with HX531 (Figure 4.8, E). As with previous experiments, no difference was found in the peak to peak amplitude or rise time of action potentials, or for changes in the RMP or input resistance of neurons between any conditions at any time point (Appendix 1.06). Taken together, these data suggest that inhibition of the RAR via the antagonist LE540 does not alter the RA-induced increases in half-amplitude duration or decay time of RA-exposed neurons. Neurons exposed to the RXR antagonist HX531, however, show half-amplitude durations and decay times of action potentials that were significantly different from controls, yet also significantly reduced compared to neurons exposed to RA alone. These data suggest that the RXR may possibly play a role in the RA-induced firing changes, as RXR impairment appears to provide a partial blockade of RA-induced changes.

In summary, experiments utilizing both RAR and RXR agonists and antagonists suggest that the RXR, but not the RAR, may be involved in RA's effects on cell firing. RAR agonist application was unable to mimic RA-exposure and, similarly, RAR impairment via antagonist incubation did not impair the RA-induced effects on cell firing. Interestingly, while RXR agonist application could not mimic RA-exposure, impairment of the RXR with an antagonist could partially block the RA-induced effects on cell firing. Thus, while the RAR appears to be unnecessary, the RXR may be required to elicit some effects of RA.

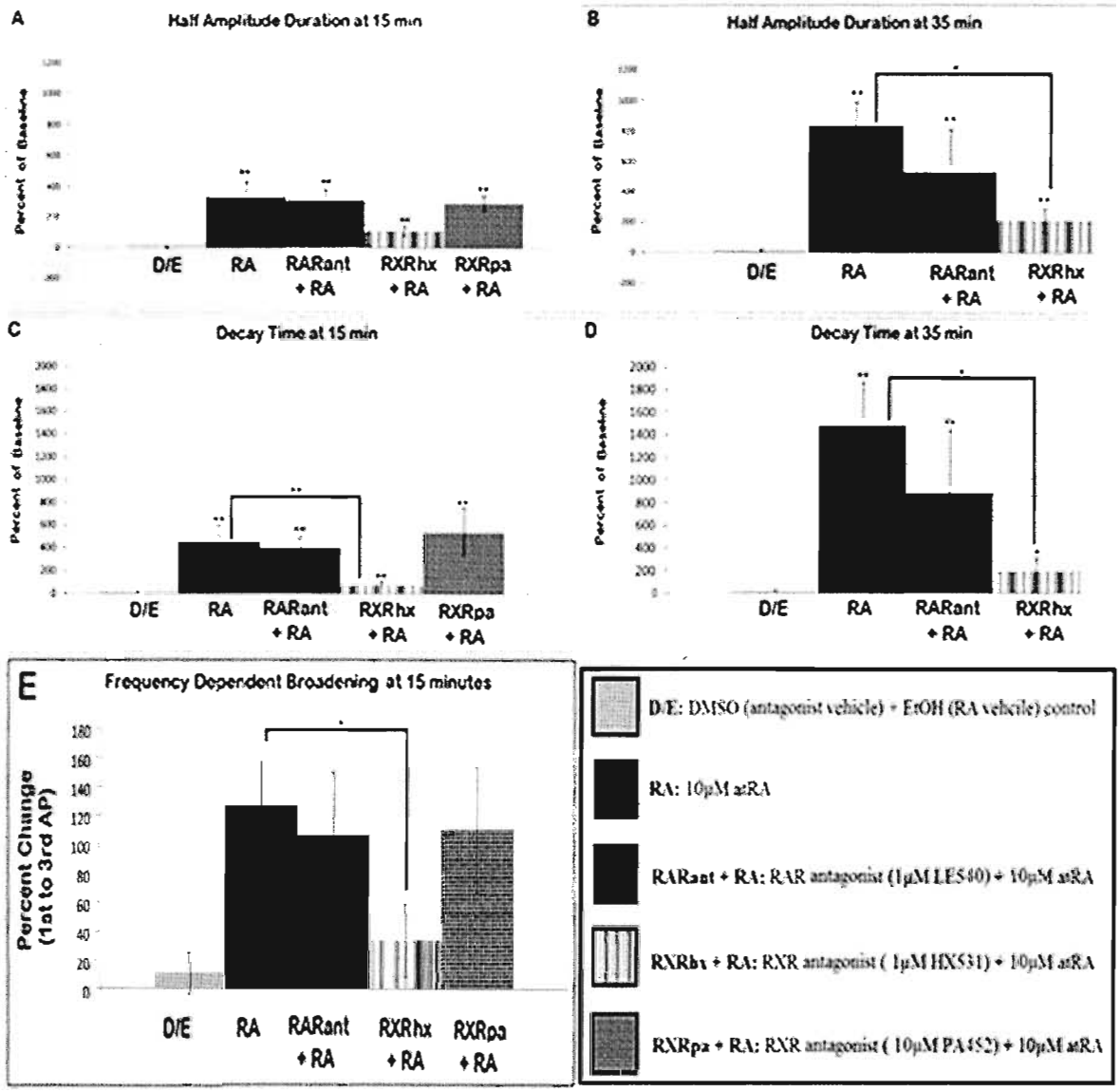


Figure 4.8. RXR inhibition, but not RAR inhibition, blocks RA-induced changes in spike waveform and frequency-dependent broadening. Neurons pre-exposed to LE540, PA452 or HX531 and then acutely exposed to RA displayed significantly higher half-amplitude durations (A & B) and decay times (C & D) when compared to controls both 15 and 35minutes of retinoid exposure (baseline set at 0%). No difference was found between LE540 or PA452-exposed neurons compared to neurons exposed to RA alone at either time point. Neurons exposed to HX531 were found to have significantly reduced half-amplitude duration and decay times compared to neurons exposed to RA alone. Neurons incubated in 1μM HX531 followed by RA exposure have significantly reduced frequency-dependent impulse broadening (E) compared to RA exposure alone. Frequency-dependent impulse broadening, expressed a percent change from the 1st action potential (set at 0%) was significantly increased in RA-exposed neurons compared to control. 15 minute replicates: control: n=11, RA: n=14, LE540: n=12, HX531: n=10, PA452: n=14; 35 minute replicates: control: n=10, RA: n=7, LE540: n=6, HX531: n=4. (*=$p<0.05$. **=$p<0.01$)

III. Acute RA exposure reduces intracellular calcium within the first hour of exposure.

Changes in intracellular calcium ($[Ca]_i$) have been shown to play a role in the firing pattern of neurons, for example by modulating bursting activity (Coulon et al., 2009; Sanchez-Alonso et al., 2010). Furthermore, alterations in $[Ca]_i$ can cause widespread physiological changes within a cell, potentially leading to an altered electrical state through the downstream modification of, for example, ion channels. Therefore, I next sought to investigate whether acute RA exposure can produce changes in $[Ca]_i$. To this end, I utilized the calcium indicator dye Indo-1 AM. This fluorescent indicator, when bound to calcium, shifts its emission spectrum, thus providing an opportunity to estimate $[Ca]_i$ by taking the ratio of emission at the optimal wavelengths for calcium-free and calcium-bound dye. This offers an advantage over other calcium imaging methods in that there is no need to control for loading differences across preparations. Furthermore, Indo-1 is fluorescently excitable only when the AM group is enzymatically cleaved by endogenous (cytosolic) esterases. Since the fluorescently excitable form of the Indo-1 dye is only present once inside neurons, this dye produces minimal background signals in the surrounding culture environment and, thus, can resolve changes in $[Ca]_i$ within cells with a high sensitivity.

In the following experiments cultured VF neurons were incubated in the presence of Indo-1 AM dye for 3 hours. Preliminary experiments revealed this incubation time to be sufficient for adequate dye loading of neurons (data not shown). After incubation, neurons were fluorescently imaged for 20 minutes prior to and 60 minutes after the addition of 10 μM atRA or EtOH (as control). In all cases, the ratio of free to bound dye during the 20 minute pre-exposure period was found to be stable (not to change significantly) with time, suggesting that dye loading and subsequent washout did not adversely affect the cells (data not shown). A baseline ratio, obtained

by taking the average of a 20 minute period immediately prior to RA or EtOH, exposure was determined, and changes in $[Ca]_i$-induced fluorescence ratios after RA or EtOH application were calculated as a percent change of this baseline.

i. RA exposure decreases $[Ca]_i$-induced fluorescence with a time course that coincides with RA-induced firing changes.

As shown in Figure 4.9, neurons exposed to 10 μM atRA (n=12) showed a significant decrease in $[Ca]_i$-induced fluorescence over time, whereas no significant difference was found with EtOH controls (n=14). Furthermore, when compared to EtOH controls at the same time point, RA-exposed neurons showed a significant decrease in intracellular calcium level as early as 10 minutes after RA application (Figure 4.9). This decrease became more dramatic over time, reaching a maximum by 45 minutes. Interestingly, the time course and magnitude of the RA-induced changes in intracellular calcium level closely resemble that seen from my previous work examining RA-induced changes in firing pattern (Chapter 3). More specifically, the RA-induced increases in half-amplitude duration and decay time of action potentials were found to be statistically significant within 15 minutes of RA exposure. These effects were found to become more dramatic over time, reaching a maximal effect approximately 35-50 minutes after RA exposure. Similarly, a significant decrease in intracellular calcium level is observed as early as 10 minutes after RA application. This decrease intensifies over time, reaching maximal effect approximately 45 minutes after RA application. These data suggest that the RA-induced decrease in intracellular calcium level coincides with the RA-induced electrophysiological changes reported previously (Chapter 2).

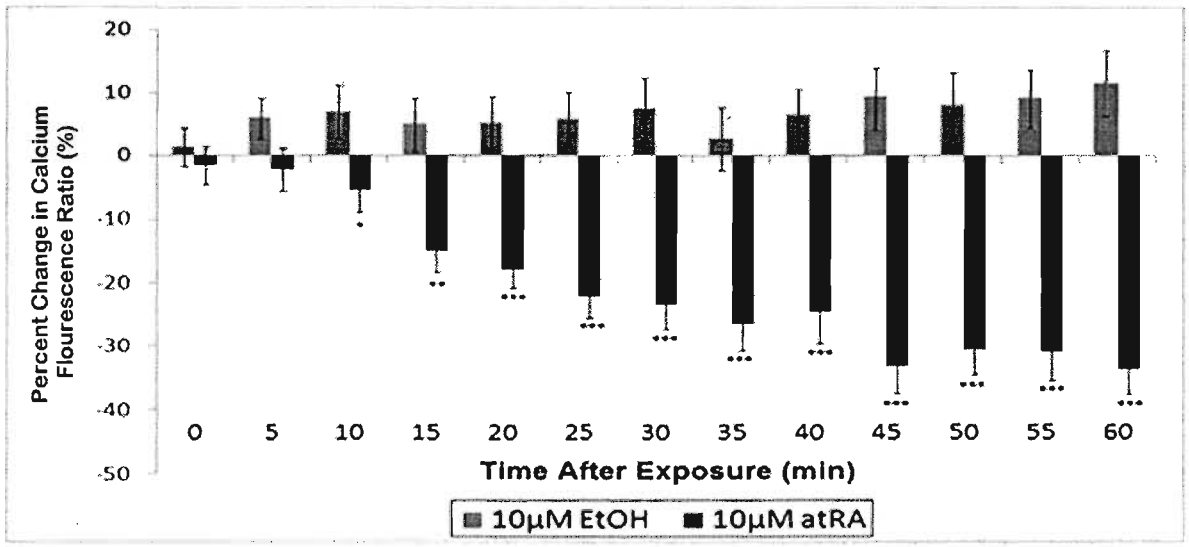


Figure 4.9. Acute RA exposure causes a rapid decrease in intracellular calcium. RA exposure caused a significant decrease in $[Ca]_i$ as early as 10 minutes after application when compared to EtOH controls at the same time point. RA was found to significantly reduce $[Ca]_i$ over time (ie: within group differences; for clarity data not shown). Data are expressed as the percent change from baseline (set at 0%). (*$p<0.05$, **$p<0.01$, ***$p<0.001$)

ii. RA-induced decreases in intracellular calcium are dose-dependent and isomer specific.

I have previously shown that the RA-induced firing changes were both dose-dependent and isomer-dependent. Having shown that exposure to 10 μM atRA produces a decrease in intracellular calcium levels with a time course similar to that of the RA-induced firing changes, I next sought to determine if this decrease is also dependent on either the retinoid dose or isomer used. In these experiments neurons were dye-loaded as performed previously, and then imaged 20 minutes prior to and for one hour after exposure to 10μM atRA, 1μM atRA, 10μM 9*cis* RA or EtOH (control).

When cells were exposed to 1μM atRA (n=15) or 10μM 9*cis* RA (n=12), there were no significant differences in intracellular calcium levels over the 60 minute duration of the experiment, and no differences were found when compared to EtOH controls (n=14). As shown previously, 10μM atRA-exposed neurons (n=12) showed a significant decrease in intracellular calcium over time. 10μM atRA-exposed neurons showed significantly lower intracellular calcium levels when compared to both 10μM 9*cis* RA and 1μM atRA -exposed neurons as early as 10 and 20 minutes after retinoid exposure, respectively (Figure 4.10). These data clearly show that a 10-fold lower concentration of atRA, or the same concentration of the 9*cis* RA isomer, did not induce similar decreases in intracellular calcium to that seen in 10μM atRA-exposed neurons. Taken together, these data suggest that, similar to the RA-induced firing changes, the RA-induced decrease in intracellular calcium appeared to be both dose-dependent and isomer-dependent.

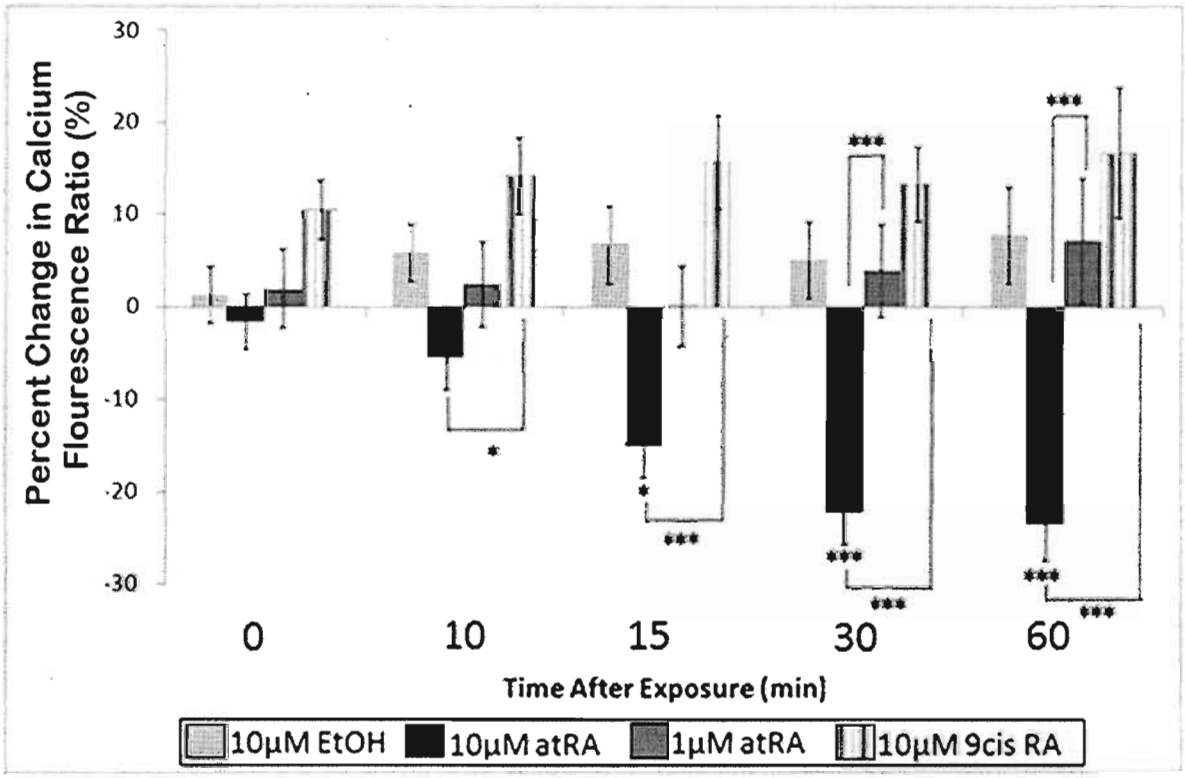


Figure 4.10. RA-induced decreases in intracellular calcium are dose-dependent and isomer-dependent. 10μM atRA-exposed neurons displayed lower calcium fluorescence ratios when compared to either 10 μM 9*cis* RA or 1μM atRA-exposed neurons, as well as controls at the same time point. For clarity, only results for 0, 10, 15, 30 and 60 minute time points after retinoid exposure are shown. Asterix over bar represents a significant difference when compared to EtOH control. Data are expressed as the percent change from baseline (set at 0%). (*$p<0.05$, **$p<0.01$, ***$p<0.001$)

iii. RXR inhibition did not block RA-induced decrease in intracellular calcium.

Having shown that the RA-induced firing changes and the RA-induced decrease in intracellular calcium share a similar time course, dose-dependency and isomer-specificity, I next sought to determine whether RA-induced changes would also be inhibited by the RXR antagonist HX531 (as are some electrophysiological changes). In the following experiments cultured VF neurons were incubated in either the RAR antagonist LE540 (n=11) or RXR antagonist HX531 (n=11) (1μM in both conditions) for one hour prior to the start of fluorescence imaging. After incubation, imaging experiments were performed as in the previous section to measure changes in calcium fluorescence ratio 20 minutes prior to and up to 60 minutes after RA exposure. Positive controls were neurons which were incubated in 0.001% DMSO for one hour and then exposed to RA (n=11). As shown in Figure 4.11, RA induced a decrease in intracellular calcium, regardless of the presence or absence of retinoid receptor antagonists, and no significant difference was found between any conditions. These data suggest that the RA-induced decrease in intracellular calcium can still occur despite inhibition of either the RAR or RXR. Interestingly, the decrease in intracellular calcium persisted in the presence of HX531, which previously partially blocked the RA-induced changes in spike waveform.

The above data suggest that RA-induced changes in spike waveform and $[Ca]_i$ may differ in some respects (dependence on RXR), although not in others (dose- and isomer-dependency), thus raising the possibility that there may be independent effects of RA. In order to investigate whether this is the case, I next aimed to utilize pharmacological agents to artificially increase or decrease $[Ca]_i$ to directly examine the effects of changing $[Ca]_i$ on spike waveform. To this end I attempted to use the calcium chelator BAPTA-AM as well as caffeine. BAPTA-AM and caffeine have previously been shown to decrease (Herring et al., 1998) and increase (Ahmed et al., 1997)

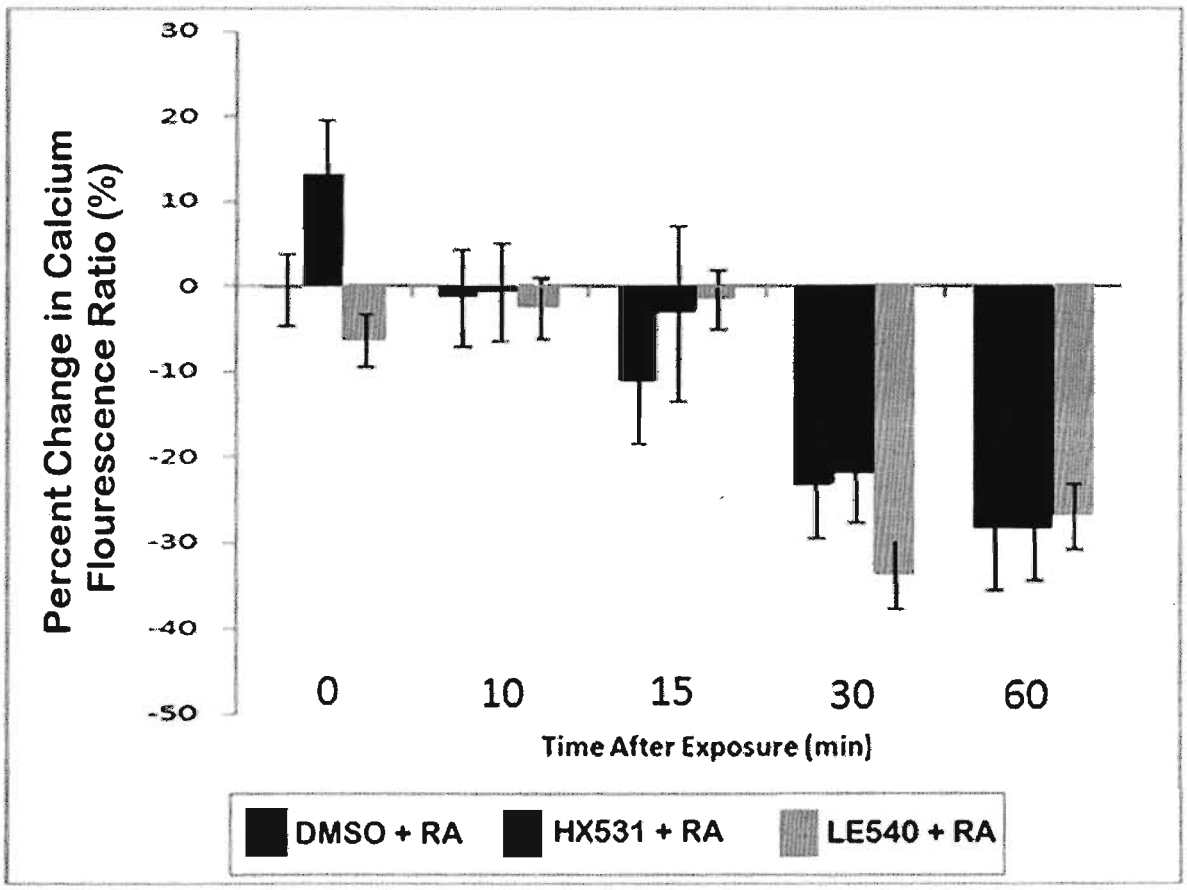


Figure 4.11. RA-induced decreases intracellular calcium are not affected by RAR or RXR antagonists LE540 and HX531. In all cases, RA-exposed neurons displayed significantly less intracellular calcium over time within each group independently (for clarity not shown). No significant differences were found between groups at any time point. Baseline was set at 0%.

$[Ca]_i$ levels in *Lymnaea* neurons, respectively. As such these reagents offer the ability to either mimic (via BAPTA-AM application) or prevent (via caffeine application) the RA-induced decrease in $[Ca]_i$.

In these experiments cultured cells were loaded with Indo-1 AM and imaged as performed previously. Initial trials utilizing 50μM BAPTA-AM indicated a reduction in the calcium fluorescence ratio that was comparable to the RA-induced decreases (Appendix 1.07); however, this BAPTA-AM -induced decrease would only occur if administered in saline containing low calcium (n=5). Unfortunately, exposure to the low calcium saline prevented any action potential firing in neurons (even after replacement with normal saline), making an investigation of spike waveform unfeasible. Similarly, preliminary experiments with caffeine did not prove to be feasible, as I was unable to detect any increase in the calcium fluorescence ratio in response to caffeine application, even at concentrations as high as 100μM (n=6) (data not shown).

IV. Inhibition of calcium-dependent K channels did not mimic RA-induced firing changes.

The observation that 10μM atRA alters intracellular calcium suggests the possibility that RA may also indirectly affect channels which are dependent upon $[Ca]_i$, such as calcium-dependent potassium channels (K_{Ca}). Many studies have previously shown that the firing properties of neurons can be dramatically altered if these channels are impaired or blocked. For example, work performed in the snail *P.trivolvis* has shown that blockade of K_{Ca} channels via application of apamin (0.6μM), caused a silencing of firing activity (Artinian et al., 2010) similar to that observed in RA-exposed neurons here. Similarly, application of 1μM apamin was able to disrupt the rhythmic firing pattern of neurons of the snail *C.atrolabiata* (Vatanparast and Janahmadi,

2009) and, in rats, led to a reduction in the afterhyperpolarization of action potentials (Shepard and Bunney, 1991). All these effects are characteristic of the RA-induced electrophysiological changes reported in Chapter 2. Some K_{Ca} channels are calcium-dependent and voltage insensitive (Artinian et al., 2010). Therefore, I hypothesized that the RA-induced decrease in $[Ca]_i$ may lead to reduced K_{Ca} channel activity, possibly producing the observed electrophysiological changes described in Chapter 2. If this is indeed the case, it would be predicted that blockade of K_{Ca} channel function, via application of apamin, would produce changes in firing pattern similar to those seen in neurons exposed to RA.

In the following experiments neurons were cultured as described previously, and were exposed to either 10μM RA or a "matching" concentration of 10μM apamin. Recordings were performed 10 minutes prior to and up to one hour after exposure to either agent. As shown in Figure 4.12, neurons exposed to 10μM RA (n=10) had a significantly increased probability of atypical impulse activity compared to neurons exposed to 10μM apamin (n=8). Additionally 70% (7/10) of neurons exposed to RA were found to become silenced within 60 minutes of exposure, whereas all neurons (8/8) exposed to apamin were able to fire action potentials for the duration of the recording (Figure 4.12, B). These data indicate that apamin does not induce RA-like changes in firing activity or cell silencing. Similarly, analysis of both the half-amplitude duration and decay time of action potentials at 15 and 35 minutes post-application as well as analysis of frequency-dependent impulse broadening showed that, in all cases, RA-exposure produced significant increases compared to apamin-exposed neurons (Figure 4.13). Taken together, these data strongly suggest that impairment of K_{Ca} channel function with apamin, at least at a 10μM concentration, did not elicit RA-like effects. Thus, it would appear that K_{Ca} channels do not appear to play a role in the RA-induced changes in firing pattern.

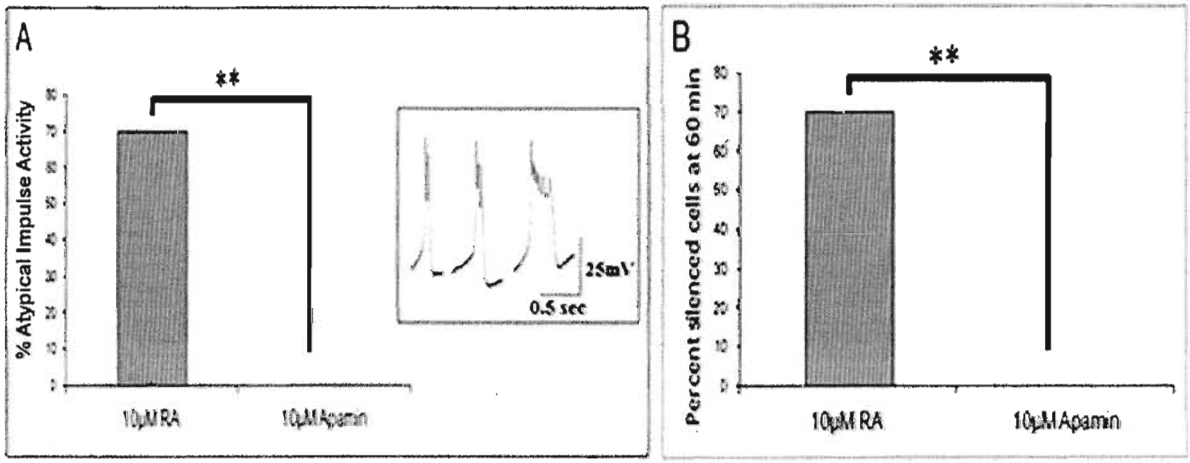


Figure 4.12. The K_{Ca} channel blocker, apamin, does not induce RA-like changes in firing activity or cell silencing. The probability of showing atypical impulse activity (A) or cell silencing (B) of RA-exposed neurons is increased compared to neurons exposed to apamin alone. (**= $p<0.01$).

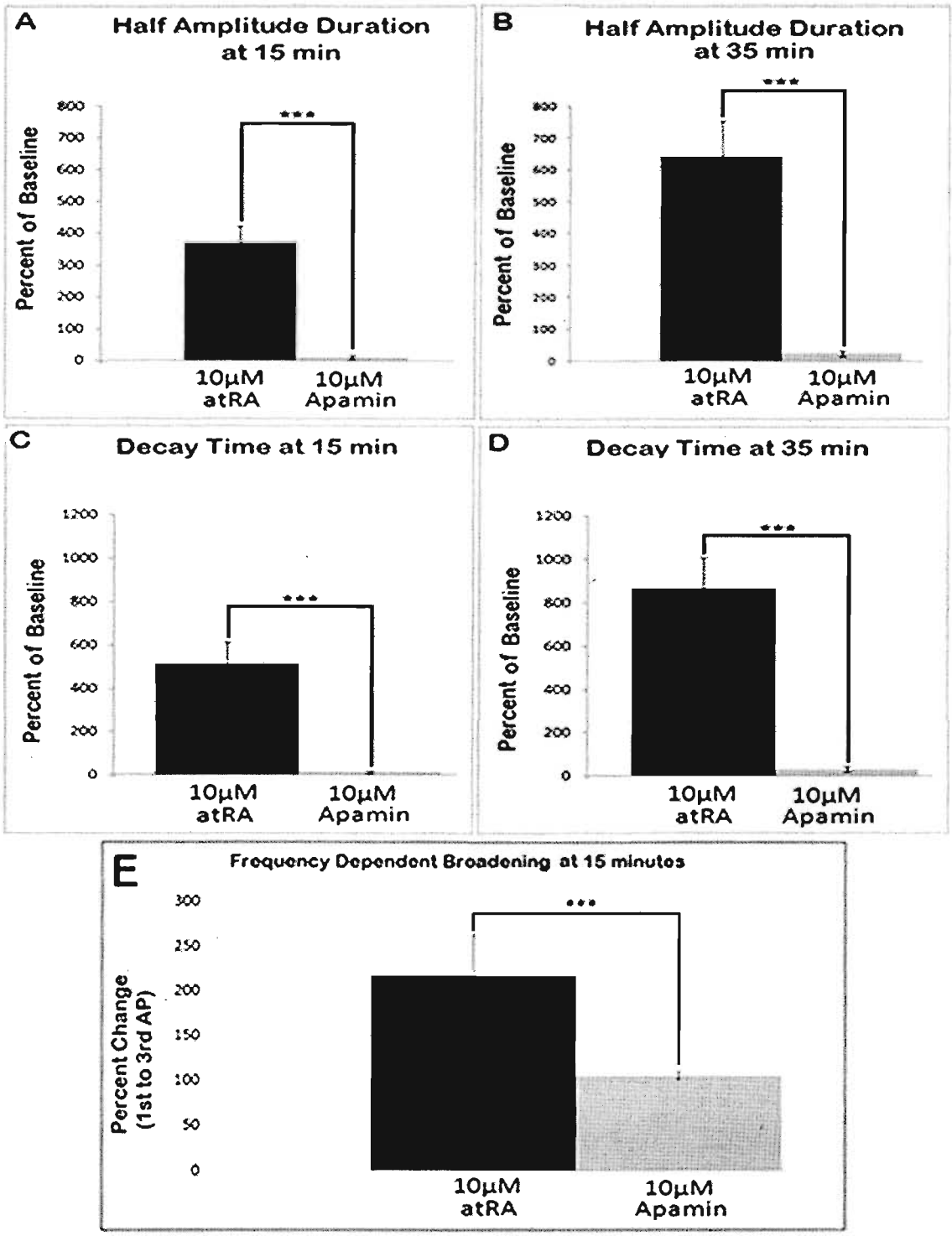


Figure 4.13. The K_{Ca} channel blocker, apamin, does not induce RA-like changes in action potential shape or frequency-dependent broadening. The half-amplitude duration (A & B) and decay time (C & D) of action potentials exposed to 10μM apamin did not exhibit RA-like increases (baseline set at 0%). In all cases neurons exposed to RA showed increased changes in half-amplitude duration and decay time of action potentials that were significantly different from apamin-exposed neurons. Apamin does not induce similar increases in frequency-dependent broadening (E) seen in RA-exposed neurons (first action potential set at 100%). (***= $p<0.01$).

4.05 - Discussion

The above work has taken the first steps toward elucidating the mechanisms by which RA elicits its effects on cell firing. Even though RA has been shown to act on protein synthesis in rat (Poon and Chen, 2008), PKA pathways in mouse (Kholodenko et al., 2007), PLC pathways in *Xenopus* (Liou et al., 2005) and calcium influx in mouse (Chen and Napoli, 2008b), in all cases the use of inhibitors here did not provide any evidence for their role in the RA-mediated effects on cell firing. Interestingly, results with the translation blocker anisomycin in particular add strong support to suggest that the classical retinoid signaling pathway is not involved. It must be taken into consideration that only one inhibitor of each pathway was used in these experiments; thus, these findings do not conclusively rule out the involvement of these pathways in a RA-mediated response. However, previous studies in other animal models have shown that similar targeted impairment using the same inhibitors used in these studies can indeed block some RA-induced effects (Kholodenko et al., 2007; Liou et al., 2005; Chen and Napoli, 2008b; Farrar et al., 2009). Moreover, the inhibitors used herein have been shown to effectively block their intended targets in *Lymnaea* at concentrations equal to or lower than those used in this thesis (Farrar et al., 2009; Hiroshi-Nakamura and Bregestovski, 1977; Lacchini et al., 2006). Moreover, the reported IC_{50} concentrations for the above inhibitors are typically at least an order of magnitude below that used in these studies (Appendix 1.08). The IC_{50} values listed in appendix 1.08 were obtained from a variety of other organisms as no values have been determined in *Lymnaea,* thus, while the concentrations of inhibitors used in this study appear to be above known IC_{50} concentrations for other organisms, continued studies determining IC_{50} values in *Lymnaea* are required to verify this speculation. With the assumption that the inhibitors were used at an effective concentration, it is likely that the targeted impairment used here would have likely reduced at least some of the RA-induced changes if RA operated through these signaling pathways. While continued efforts at examining other target proteins within each signaling

pathway will be required to strengthen these findings, these data indicate that these pathways are not likely involved in the RA-induced firing changes.

Interestingly, while 10μM cadmium treatment appeared to elicit a partial block of several RA-induced changes (Figures 4.2 & 4.3), no such blockade was observed with a higher (30μM) cadmium concentration (Figure 4.4). While a 10μM cadmium concentration has been previously used to block growth cone turning in *Lymnaea* (Farrar et al., 2009), blocking electrophysiological effects in *Lymnaea* has required concentrations as high as 50μM (Molnar et al., 2004). Thus, different cellular responses (morphological vs. electrophysiological) may be differentially sensitive to the targeted blockade of voltage-gated calcium channels. Currently it is unclear why a lower concentration of cadmium would appear to provide a partial blockade of the RA-induced changes. However, I have provided strong evidence with the higher concentration to suggest that calcium influx through voltage-gated calcium channels may not be required to elicit RA's effects.

When investigating the possibility that the retinoid receptors may play a role in the RA-induced firing changes, my data strongly suggest that the RAR is not involved. Exposure to an agonist selective for the RAR was not able to mimic the RA-induced changes in atypical impulse activity, cell silencing or changes in spike waveform. Moreover, application of an RAR-selective antagonist was unable to block any of the RA-induced changes in firing pattern. Taken together these data suggest that the RAR is not required to elicit the RA-induced changes in firing pattern. Thus, these findings may rule out the possibility that RA elicits its electrophysiological effects through the RAR, either classically to activate gene transcription, or non-genomically. As will be

discussed in a following section, a remaining question needing to be addressed is the appropriateness of the agonists and antagonists used in these experiments.

When focusing on the role of the RXR, the interpretation of my data proves more difficult. The data from PA024 and PA452-treated cells are complementary; the RXR agonist PA024 did not induce RA-like effects, and blocking the RXR with PA452 did not impair RA-induced changes. In the absence of any other conditions, one would come to the conclusion that the RXR, like the RAR, is probably not be involved in the RA-induced effects. Interestingly, neurons treated with the RXR antagonist HX531 showed significant impairments in the RA-induced effects. In interpreting these data, one must call into question not only whether PA024 is sufficiently activating the RXR, but additionally whether PA452 is sufficiently inhibiting the RXR. While PA024 was used at a concentration of 10μM to "match" the concentration of RA, it is possible that PA024 may not be as effective as RA at activating the RXR. I have previously shown dose-dependent responses to RA such as increases in half-amplitude duration and decay time. Thus, (assuming that the RXR is indeed involved) a less effective activation of the RXR at the same concentration induced by PA024 (when compared to that induced by RA) could be insufficient to cause the RA-like effects on firing pattern. In other words, if PA024 is not as effective as RA at activating the RXR, this "matched" concentration of agonist would resemble a lower dose of RA, which I have shown does not induce RA-like firing changes. Similarly, it is unclear whether PA452 is inhibiting the RXR sufficiently. Previous work in our lab has shown that HX531 may be a more effective antagonist than PA452 (Carter et al., 2010). Thus, the results obtained here may suggest that PA542 may only offer a partial impairment of the RXR, and HX531 may offer a more severe impairment; however, continued studies are required to verify this claim. It is tempting to speculate that the RXR may not be involved in the RA-induced firing changes, as

this provides the simpler explanation wherein only the effectiveness of HX531is called into question, rather than both PA024 and PA452. Currently, the only means to elucidate the involvement of the RXR in the RA-induced firing effects lies in the validation of the agonists and antagonists being used. Thus at present, the role (if any) of the RXR has yet to be elucidated.

The results pertaining to the RXR directly call into question the selectivity of the agonists and antagonists being used in the present experiments. While extensively used in other vertebrate models (Bianchi et al., 2009; Del Rincon and Scadding, 2002; Konta et al., 2001; Kagechika, 2002; Suzuki et al., 2009; Honda et al., 2005), it is important to note that with the exception of recent work in our lab (Carter et al., 2010), no other studies have documented use of these reagents in *Lymnaea* or any other invertebrate. Thus, it is unclear whether these pharmacological agents have the same binding affinity and selectivity for *Lymnaea* retinoid receptors as they do for their vertebrate counterparts. While not conclusively validated, there are currently two lines of evidence which suggest that these agonists/antagonists may have the similar selectivity in *Lymnaea* as seen in other vertebrates. First, the RAR and RXR ligand binding domains, which would be the targeted area for the agonists/antagonists used here, share approximately 58% amino acid identity with human RARβ (Carter, 2011) and 81% amino acid identity with rat RXRα, respectively (Carter et al., 2010). Moreover, both receptors share a 100% amino acid identity at the specific residues which are thought to interact with RA in the active site (Carter, thesis). These data suggest that due to their homology with other vertebrate retinoid receptors (which have validated the use of these agonists/antagonists), *Lymnaea* retinoid receptors are likely targets of these reagents. Secondly, use of these agonists/antagonists in our model system has produced similar results to those seen in other vertebrate animals utilizing the same reagents. For example, TTNPB and LE540 have been previously used in our lab and have been found to

disrupt developmental processes leading to deformities of *Lymnaea* embryos (Carter et al., 2010). These deformities are similar to the perturbations typically seen when a developing embryo is exposed to excessive RA levels and suggest that these agents can indeed disrupt the RA signaling pathway. Similarly, the RXR agonist PA024 has been previously used in our lab and has been found to elicit positive growth cone turning of neurites in culture in a similar fashion to that seen from application of RA (Farrar et al., 2009). Moreover, such PA024-induced growth cone turning can be impaired in the presence of both HX531 and PA452 (Carter et al., 2010), suggesting that both RXR antagonists can impair the PA024-induced effects. Lastly, as shown in Appendix 1.08, the concentration of agonists and antagonists used within the current studies are typically at least an order of magnitude higher than that of reported EC_{50} and IC_{50} values for other organisms. Since no such values are known for *Lymnaea*, these values should be considered as a general range of effective concentration, rather than absolute value. While it is possible that in *Lymnaea* the EC_{50} and IC_{50} values of the agonists or antagonists may differ, it is reasonable to assume that at the concentrations used here, one would have elicited at least a partial activation (in the case of an agonist) or impairment (in the case of an antagonist) of the retinoid receptors, as well as possibly eliciting nonselective effects. Thus, while conclusive binding studies are required to confirm the selectivity and compare the binding affinities with natural ligands in *Lymnaea*, these data provide indirect evidence that the RAR/RXR agonists can induce RA-like effects, and that RAR/RXR antagonists can impair RA-induced effects.

I next sought to determine if RA exposure resulted in alterations to $[Ca]_i$. Transient changes in $[Ca]_i$ have been shown to induce bursting activity in neurons (Coulon et al., 2009) as well as play a role in modulating burst duration (Sanchez-Alonso et al., 2010). Moreover, RA exposure has been shown to cause modest changes in $[Ca]_i$ in both rat and human cultured cell lines (Short et

al., 1991; Gao et al., 1998). More specifically, Short *et al.* (1991) have shown that cells pretreated with RA can exhibit dramatic increases in $[Ca]_i$ in response to hormone exposure in culture. Similarly, Gao *et al.* (1998) have shown that RA-induced cell differentiation results in changes in $[Ca]_i$ as cells mature. In both cases, any RA-induced changes are confounded by other factors in these experiments, and thus a direct examination into whether RA can alter $[Ca]_i$ levels has not been performed. I have now shown that acute exposure to RA results in a significant decrease in $[Ca]_i$ levels as early as 10 minutes after application. Interestingly the time course, dose-dependency and isomer-dependency of this $[Ca]_i$ decrease closely resemble those reported in my previous work (Chapter 3) characterizing the electrophysiological responses to RA. It has been suggested that alterations in the firing pattern of injured neurons may act to create a favorable intracellular environment, containing less intracellular calcium, to promote the regeneration of neurons (McClellan et al., 2008). My work has shown that RA does indeed alter the firing pattern of neurons, and that $[Ca]_i$ levels decrease correspondingly. I speculated in Chapter 3 that RA may elicit its regenerative effects by transiently promoting neurite sprouting in part by altering firing pattern and reducing calcium levels within the cell. These data suggest that this may be the case. While not attempted here, it would be beneficial to determine in future studies whether $[Ca]_i$ increases after 3 hours of RA exposure, corresponding to the return of firing activity. Taken together, these data compliment the electrophysiological effects of RA and, to my knowledge, represent the first studies performed documenting a direct RA-induced decrease in $[Ca]_i$. These results also suggest that the two phenomena, electrophysiological changes and a $[Ca]_i$ decrease, may be linked.

In an attempt to determine if the electrophysiological changes seen were responsible for the decrease in intracellular calcium, I sought to determine whether the $[Ca]_i$ decrease occurs

downstream of the RXR. My work utilizing the RXR antagonist HX531 has shown that in the presence of this antagonist, the RA-induced changes can be partially blocked or impaired. I had hypothesized that if the RXR operates upstream of the $[Ca]_i$ decrease, then impaired RA-induced firing changes would be accompanied by a reduced $[Ca]_i$ decrease. Interestingly, HX531 was unable to impair the decrease in $[Ca]_i$, which could suggest one of two possibilities. First, the RXR may not be operating upstream of the $[Ca]_i$ decrease, and rather the $[Ca]_i$ decrease may in fact ultimately lead to the activation of the RXR and the accompanying electrophysiological changes induced by RA. Alternatively, it is possible that exposure to RA may elicit multiple independent responses. Substantial work has shown that modification of $[Ca]_i$ can initiate a wide array of signaling cascades (Wiegert and Bading, 2010; Fields et al., 2005). This hypothesis would be in agreement with my previous work showing that HX531 treatment only partially blocks the electrophysiological response rather than completely abolishing it. If the RXR was only one of many signaling cascades which respond in parallel to RA exposure, then blocking the RXR may only partially impair the RA-induced changes in firing pattern, as shown in my current work. Further clarification into either of these speculations will require continued studies.

Lastly, I had hypothesized that the RA-induced $[Ca]_i$ decrease may have altered the ability of K_{Ca} channels to function normally. These channels open in response to transient changes in calcium concentration, and as such a reduced $[Ca]_i$ could presumably reduce the activity of these channels. I attempted to mimic the possible RA-induced impairment of K_{Ca} channels via application of apamin which selectively blocks K_{Ca} channels (Harris-Warrick and Johnson, 1987; Shepard and Bunney, 1991; Vatanparast and Janahmadi, 2009). My data suggest that apamin was not able to induce RA-like changes in firing properties, despite utilizing a concentration well in excess of the reported IC_{50} value (Appendix 1.08). While apamin has not been previously used in

Lymnaea, it has been successfully used in a wide variety of vertebrate (Coulon et al., 2009; Shepard and Bunney, 1991) and invertebrate preparations including lobster (Harris-Warrick and Johnson, 1987), lamprey (McClellan et al., 2008) and both *Planorbella* (Artinian et al., 2010) as well as *Caucasotachea* (Vatanparast and Janahmadi, 2009) snails. While these studies have typically used apamin concentrations of 1μM or less, I utilized a 10μM apamin concentration in an attempt to ensure at least a partial impairment of K_{Ca} channels. Additionally, transcriptome analysis of the *Lymnaea* CNS reveals the presence of K_{Ca} channel transcripts (Feng et al., 2009). Thus, while it has not been conclusively proven that apamin does block K_{Ca} channels in *Lymnaea,* it is reasonable to assume that these channels are present in the CNS and that, like other preparations, these channels are sensitive to apamin. While at this time I cannot rule out the possibility that the concentration of apamin used was ineffective or that K_{Ca} channels may not be expressed in VF neurons, it would appear from my current studies that despite a decrease in $[Ca]_i$, the activity of K_{Ca} channels does not appear to account for the RA-induced changes in firing properties. Continued studies would be required to verify this claim.

In summary I have provided the first evidence that the RA-induced effects on the electrophysiological properties of neurons appears to be operating outside that of the classical retinoid signaling pathway and do not require protein synthesis, the activation of PKA or PLC, or calcium influx. I have presented evidence to suggest that the RAR is not involved, but interestingly provide data suggesting a possible non-genomic role for the RXR. I show that RA can induce a decrease in intracellular calcium. It is unclear whether the electrophysiological changes and decreased intracellular calcium are directly linked and if so, which occurs upstream of the other. However, I have shown that if the decreased intracellular calcium does cause the electrophysiological changes, it does not likely do so by modulating the activity of K_{Ca} channels.

5.0 Conclusions and Perspectives

The overall aim of my study was to investigate RA's regenerative effects at the morphological and molecular levels by utilizing an *in situ* injury paradigm, and to determine whether RA can alter the firing properties of neurons. Utilizing the pond snail *Lymnaea stagnalis*, I showed for the first time that direct application of RA can increase neurite outgrowth of dopaminergic cells. Moreover, I show that this RA-induced morphological effect is contingent upon the application of a nerve-crush injury, showing that RA-exposure results in the targeted regeneration and/or protection of injured dopaminergic neurons.

Using electrophysiological recording, I showed that acute exposure to RA results in transient changes to action potential shape, eventually leading to inhibition of cell firing. I extended these studies and further showed that the RA-induced responses are both dose-dependent and isomer-dependent. Lastly, I showed that RA exposure causes a dramatic decrease in intracellular calcium levels with a similar time course, dose-dependency and isomer-dependency to those of the RA-induced changes in firing pattern. The studies provided within this thesis offer important evidence highlighting RA's ability to operate outside the confines of its classically studied pathway of transcriptional activation. Importantly, this work underscores the need for continued studies to consider RA not only in the context of a transcriptional activator, but in a number of non-transcriptional roles as well.

RA-induced Regeneration

Previous work in both vertebrate and invertebrate animal models has shown the ability for RA to act as a regeneration-inducing molecule. Application of RA, or RA analogues can lead to the

induction and enhancement of neurite outgrowth in culture (Dmetrichuk et al., 2005; Dmetrichuk et al., 2006a), directed guidance of regenerating neural tissue both in culture (Carter et al., 2010; Dmetrichuk et al., 2005; Dmetrichuk et al., 2006a; Dmetrichuk et al., 2008; Farrar et al., 2009; Katsuki et al., 2009) as well as *in situ* (Agudo et al., 2010; So et al., 2006; Ulusoy et al., 2011), and remarkably, functional recovery *in vivo* (Agudo et al., 2010; Ulusoy et al., 2011). Given that RA is thought to play a role in a number of diseases, such as Parkinson's disease (Krezel et al., 1998), schizophrenia (Krezel et al., 1998) and depression (Bremner and McCaffery, 2008), the ability for RA to act as a regeneration-inducing molecule makes it an excellent candidate for therapeutic research. Despite its potential as a therapeutic agent (Theodosiou et al., 2010; Maden and Hind, 2003; Bremner and McCaffery, 2008), very little is known about the exact role RA plays during regeneration. RA's effects are complex and widespread, as is evident in the ever growing body of literature documenting RA's ability to elicit effects outside that of the classical retinoid signaling pathway (eg: Farrar et al., 2009; Chen and Napoli, 2008b; Dmetrichuk et al., 2008; Dmetrichuk et al., 2006; Bremner and McCaffery, 2008). A molecule with what was thought to be a well-studied mechanism of action (ie: transcriptional activation of RARE containing genes), RA has rapidly emerged as a multifaceted molecule with the ability to affect many signaling pathways, both locally within cellular microdomains (Carter et al., 2010; Chen et al., 2008; Farrar et al., 2009; Han et al., 2009; Maghsoodi et al., 2008; Poon and Chen, 2008; Sidell et al., 2010) and more widespread throughout a cell (Arcangeli et al., 1998; Romero-Sandoval et al., 2006; Tonini et al., 1999; Xiao et al., 1998). Thus, the use of RA as a therapeutic agent critically depends on an understanding of the fundamental interactions this molecule has, not only during regeneration, but in the normal functioning of the nervous system. Thus "basic" research characterizing the effects RA has on regeneration is essential to our understanding of RA's many interactions, and possible applications.

Does RA's electrophysiological effects promote regeneration?

From these studies it is currently unclear exactly how RA induces regeneration at the morphological level. However, I have shown substantial evidence that RA also causes dramatic changes to the firing activity of cultured neurons. Since neurons grown in cell culture, if given the appropriate conditions, will extend neurites in an attempt to reestablish contact with their synaptic partners (Dmetrichuk et al., 2006a; Hamakawa et al., 1999), I have considered cell culture to represent an injury-induced model, and as such considered a cultured neuron to be in an injured state. Interestingly, in cell culture I have shown that RA can dramatically decrease intracellular calcium levels. Importantly, it has been suggested that changes in the firing pattern and $[Ca]_i$ of injured neurons are critical to promote regeneration following injury (Connor, 1986; McClellan et al., 2008). Specifically, it has been suggested that reduced firing activity following injury may decrease $[Ca]_i$ levels, which is known to promote neurite outgrowth. Previous work has shown that calcium influx from action potential firing (Ibarretxe et al., 2007a; McCobb et al., 1988; Cohan et al., 1987), calcium transients (Lautermilch and Spitzer, 2000) as well as exposure to a calcium ionophore (Mattson and Kater, 1987; Mattson et al., 1988b) can impair neurite extension, and thus inhibit regeneration. Moreover, some studies have also shown that blockade of calcium influx (Mattson et al., 1988a; Mattson et al., 1988b; Mattson and Kater, 1987) or application of agents shown to reduce calcium (McCobb et al., 1988) can promote neurite elongation. It is tempting to speculate that it would be advantageous for a neuron to inhibit firing following an injury so as to decrease $[Ca]_i$ levels and allow for the activation of the pathways required for the induction and sprouting of neurites. However, as electrical activity (Lautermilch and Spitzer, 2000; Ibarretxe et al., 2007a; Ming et al., 2001) and calcium influx (Farrar et al., 2009) have also been shown to play a role in axonal guidance, it would appear that at some point after the induction of neurite outgrowth, firing activity might be required to guide regenerating neurites to their synaptic targets. Thus, cell silencing may only be required transiently, providing

a short term window wherein inhibition of cell firing is beneficial to the induction of regenerative responses. While purely speculative, my data show support for this hypothesis, as RA exposure results in transient cell silencing and decreases in intracellular calcium, after which cell firing is restored to normal, pre-exposure conditions. From these data it becomes apparent that one possible explanation for how RA induces neurite outgrowth could be through the transient inhibition of cell firing, coupled with the decrease in $[Ca]_i$, which combined, create a favorable environment in which to initiate outgrowth. Once a regenerative response has been initiated, cell firing is restored to normal to aid in the guidance of extending neurites. Clearly continued studies would be required to verify this speculation.

Why is atRA more effective than 9cis RA?

My work has shown that the RA-induced effects on cell firing are isomer-dependent, as the atRA isomer can produce significantly more dramatic effects than the same concentration of the 9*cis* RA isomer. This result is interesting as typically 9*cis* RA is found to have substantially greater (more than 10x) effects when compared to the atRA isomer in other animal models (Han et al., 1995; Thaller et al., 1993). Moreover, work performed in our lab utilizing *Lymnaea* up until this point has suggested that both isomers can equally induce, enhance and direct neurite outgrowth (Dmetrichuk et al., 2008). Most work done to date examining the non-genomic effects of RA which operate independently of the retinoid receptors (such as activation of PKA, PLC, etc.) focus on atRA only (Liou et al., 2005; Al Wadei and Schuller, 2006; Zhang and McMahon, 2000; Liao et al., 2004; Canon et al., 2004; Dmetrichuk et al., 2006a), and as such, to my knowledge, 9*cis* RA has not been studied for its non-genomic, retinoid receptor-independent actions. In *Lymnaea*, 9*cis* RA-induced growth cone turning appears to require the non-genomic action of the RXR (Carter et al., 2010); however, the role of the RXR in my work remains

unclear. It is possible that RA may be operating through a non-genomic, retinoid receptor-independent mechanism, which may not utilize the 9*cis* RA isomer. If this is case, it might be expected that changes in the firing properties of neurons would be predominantly atRA-mediated. My data are in agreement with this, as I show virtually atRA-exclusive effects; however continued studies will be required to validate this claim.

Through what pathway is RA operating?

My work has shown, for the first time, an RA-induced effect on the electrophysiological properties of adult neurons. In general, although there is some evidence to implicate the RXR (which remains unconfirmed), I have found no evidence to suggest that RA operates through protein synthesis, PKA or PLC activation, calcium influx or through the RAR. These results are particularly interesting as the above mentioned pathways are well studied for their non-genomic interactions with RA and were thought to represent the most likely pathways through which RA may operate to elicit its effects. A consistent theme in this research has been highlighting the plethora of pathways that RA has been implicated to activate. As such, new work is continually emerging providing evidence for new, previously unknown interactions of RA with specific proteins or signaling pathways within cells. Continued studies would benefit from expanding the current list of pathways to include such proteins as PKC (Ochoa et al., 2003; Oh et al., 2010) the MEK1/2-ERK1/2 pathway (Zanotto-Filho et al., 2008; Gupta et al., 2008), cyclin-dependent kinase (Borriello et al., 2006) and possibly alternative splicing (Laserna et al., 2009). These pathways, and surely many others, are emerging as potential candidates through which RA can operate, and thus extending current studies to include such pathways could provide useful insights into the means by which RA operates to elicit its electrophysiological effects.

Can RA concentration reach micromolar levels in vivo?

Typically, RA concentrations in the nanomolar range have been thought to be physiologically relevant, owing largely to the quantification of endogenous RA levels during limb development and regeneration. Such work calls into question whether the higher 10μM concentration of RA (which I have used) is physiologically relevant or whether such high endogenous concentrations can be reached *in vivo*. While efforts to quantify the levels of RA in the developing limb have provided valuable insight, it should be noted that such work highlights the need to consider the reported values as a minimum level only (Scadding and Maden, 1994; Chen et al., 1994). This comes first from the inability to determine the peak level of RA at the extreme posterior end of the limb, since large limb sections (eg: the anterior half vs. the posterior half) are required as starting material in quantifying RA levels, thus diluting the maximum RA level at the limb extremity. Secondly, it has been estimated that reported RA levels can be up to 30% lower than actual tissue values due to the loss of RA during the quantification procedure (Scadding and Maden, 1994). Thus, while previous studies clearly show the presence of a RA gradient within the developing limb, it is currently unclear how high the maximum concentration of RA within this gradient might reach, and thus an estimate of the true physiological upper limit of RA concentration *in vivo* has yet to be determined. There have been reported cases where micromolar levels of RA have been found *in vivo* (De et al., 1992) and where micromolar levels of RA are required to elicit cell differentiation (Quenech'Du et al., 1998). Thus it might seem reasonable to increase the endogenous "working range" of RA to include micromolar amounts.

Is RA used in the normal, uninjured CNS?

While I have focused on RA's ability to elicit changes in action potential shape and firing behaviour, a larger issue is what effect such changes have within neuronal networks and the CNS

at large. Work performed by White et al. (2007), along with many other studies, has shown that the expression of RALDH and CYP26 (for example) can be tightly regulated to influence the synthesis or breakdown of RA locally within tissues. The ability for RA concentration to be locally altered within specific regions of tissue highlights the possibility that RA may be potentially acting in a cell-specific or synapse-specific manner to elicit targeted effects. For example, RA may be able to elicit changes in the firing properties of specific neurons which have increased RALDH expression or lowered CYP26 expression, allowing for the local accumulation of RA, potentially to micromolar levels, which I have shown are required for eliciting changes in firing pattern. Thus, RA may be able to elicit targeted effects within the CNS, either with respect to single cells producing adequate levels of RA, or potentially at synapses with adequate RA concentrations within the synapse.

Since RA alters the firing properties of neurons it is possible that RA may change the way neurons communicate and function within complex neural networks. The potential ability for RA to elicit changes in neuronal communication has widespread implications, not only in the context of development and regeneration, but also in the context of normal neuronal functioning within an uninjured, adult CNS. If, as discussed above, RA can be targeted locally to alter neuronal communication, it may play a role in modulatory responses within the CNS. This possibility extends the role of RA outside that of development and regeneration, and places it a role similar to that of a signaling molecule or neurotransmitter, which are used in the normal functioning and modulation of the nervous system. Some work has shown that RA can act in a similar fashion to that of a signaling molecule in the uninjured adult CNS, particularly in learning and memory. For example, work has shown that RA has the ability to modulate the activity of a number of genes needed in an immediate early response to a learning paradigm (Aggarwal et al., 2006; Kim et al.,

2007; Chen and Napoli, 2008a; Kampmann and Mey, 2007). Without RA's influence on these targets, learning and memory is severely impaired in the adult. Moreover, vitamin A deficient diets (Mao et al., 2006; Cocco et al., 2002) or mutation of retinoid receptors (Chiang et al., 1998; Wietrzych et al., 2005) have long been known to result in learning and memory deficits in otherwise normal adults. Given that learning and memory are largely thought to involve changes in synapses and neuronal communication, these studies show that RA can potentially serve as a modulator in synapse formation and/or neuronal communication in the adult. My work suggests that RA may play a role in modulating neural activity which ultimately might change neural communication. Taken together, these data suggest the possibility that in the adult, RA may take on other roles in the adult CNS, potentially as a signaling molecule influencing the activity of neurons within neural networks.

Perspectives.

RA has long been known to play important roles in development and patterning, through what has now become an extensively well studied retinoid signaling pathway. Until recently, RA was thought to operate exclusively through transcriptional activation. In recent years the roles RA is known to play, particularly in regeneration, have become increasingly widespread, as new evidence is emerging showcasing RA's ability to elicit effects in novel, previously undocumented ways that extend well beyond that of transcriptional activation. Moreover, RA is continually being implicated in roles other than development and regeneration. In this study I sought to determine the morphological response to RA in a nerve-crush injury paradigm and to investigate the possibility that RA can alter the electrophysiological properties of neurons. My data highlight new, novel effects of RA on regenerating neurons as well as RA's ability to

influence the firing activity of neurons, which further widens RA's range of abilities and opens new avenues for continued research.

Reference List

Aggarwal,S., Kim,S.W., Cheon,K., Tabassam,F.H., Yoon,J.H., and Koo,J.S. (2006). Nonclassical action of retinoic acid on the activation of the cAMP response element-binding protein in normal human bronchial epithelial cells. Mol. Biol. Cell *17*, 566-575.

Agudo,M., Yip,P., Davies,M., Bradbury,E., Doherty,P., McMahon,S., Maden,M., and Corcoran,J.P. (2010). A retinoic acid receptor beta agonist (CD2019) overcomes inhibition of axonal outgrowth via phosphoinositide 3-kinase signalling in the injured adult spinal cord. Neurobiol. Dis. *37*, 147-155.

Ahlemeyer,B. and Krieglstein,J. (2000). Inhibition of glutathione depletion by retinoic acid and tocopherol protects cultured neurons from staurosporine-induced oxidative stress and apoptosis. Neurochem. Int. *36*, 1-5.

Ahmed,I.A., Hopkins,P.M., and Winlow,W. (1997). Low concentrations of caffeine raise intracellular calcium concentration only in the presence of extracellular calcium in cultured molluscan neurons. Gen. Pharmacol. *28*, 245-250.

Al Wadei,H.A. and Schuller,H.M. (2006). Cyclic adenosine monophosphate-dependent cell type-specific modulation of mitogenic signaling by retinoids in normal and neoplastic lung cells. Cancer Detect. Prev. *30*, 403-411.

Aldrich,R.W., Jr., Getting,P.A., and Thompson,S.H. (1979). Mechanism of frequency-dependent broadening of molluscan neurone soma spikes. J. Physiol *291*, 531-544.

Allenby, G., Bocoquelt, M., Saunders, M., Kazmer, S., Speck, J.,Rosenberger, M., Lovely, A., Kastner, P. and . Levin, A. (1993). Retinoic acid receptors and retinoid X receptors: Interactions with endogenous retinoic acids. P.N.A.S. (90):30-34

Arcangeli,A., Rosati,B., Cherubini,A., Crociani,O., Fontana,L., Passani,B., Wanke,E., and Olivotto,M. (1998). Long-term exposure to retinoic acid induces the expression of IRK1 channels in HERG channel-endowed neuroblastoma cells. Biochem. Biophys. Res. Commun. *244*, 706-711.

Artinian,L., Tornieri,K., Zhong,L., Baro,D., and Rehder,V. (2010). Nitric oxide acts as a volume transmitter to modulate electrical properties of spontaneously firing neurons via apamin-sensitive potassium channels. J. Neurosci. *30*, 1699-1711.

Bianchi,M.G., Gazzola,G.C., Cagnin,S., Kagechika,H., and Bussolati,O. (2009). The ATRA-dependent overexpression of the glutamate transporter EAAC1 requires RARbeta induction. Biochim. Biophys. Acta *1788*, 1861-1868.

Bonet,M.L., Puigserver,P., Serra,F., Ribot,J., Vazquez,F., Pico,C., and Palou,A. (1997). Retinoic acid modulates retinoid X receptor alpha and retinoic acid receptor alpha levels of cultured brown adipocytes. FEBS Lett. *406*, 196-200.

Borriello,A., Cucciolla,V., Criscuolo,M., Indaco,S., Oliva,A., Giovane,A., Bencivenga,D., Iolascon,A., Zappia,V., and Della,R.F. (2006). Retinoic acid induces p27Kip1 nuclear accumulation by modulating its phosphorylation. Cancer Res. *66*, 4240-4248.

Bremner,J.D. and McCaffery,P. (2008). The neurobiology of retinoic acid in affective disorders. Prog. Neuropsychopharmacol. Biol. Psychiatry *32*, 315-331.

Carter, C. (2011) Identification of novel retinoid receptors and their roles in vertebrate and invertebrate nervous systems. PhD Thesis, Brock University.

Canon,E., Cosgaya,J.M., Scsucova,S., and Aranda,A. (2004). Rapid effects of retinoic acid on CREB and ERK phosphorylation in neuronal cells. Mol. Biol. Cell *15*, 5583-5592.

Carter,C.J., Farrar,N., Carlone,R.L., and Spencer,G.E. (2010). Developmental expression of a molluscan RXR and evidence for its novel, nongenomic role in growth cone guidance. Dev. Biol. *343*, 124-137.

Chen,N. and Napoli,J.L. (2008a). All-trans-retinoic acid stimulates translation and induces spine formation in hippocampal neurons through a membrane-associated RARalpha. FASEB J. *22*, 236-245.

Chen,N. and Napoli,J.L. (2008b). All-trans-retinoic acid stimulates translation and induces spine formation in hippocampal neurons through a membrane-associated RARalpha. FASEB J. *22*, 236-245.

Chen,N., Onisko,B., and Napoli,J.L. (2008). The nuclear transcription factor RARalpha associates with neuronal RNA granules and suppresses translation. J. Biol. Chem. *283*, 20841-20847.

Chen,Y., Huang,L., and Solursh,M. (1994). A concentration gradient of retinoids in the early Xenopus laevis embryo. Dev. Biol. *161*, 70-76.

Chiang,M.Y., Misner,D., Kempermann,G., Schikorski,T., Giguere,V., Sucov,H.M., Gage,F.H., Stevens,C.F., and Evans,R.M. (1998). An essential role for retinoid receptors RARbeta and RXRgamma in long-term potentiation and depression. Neuron *21*, 1353-1361.

Cocco,S., Diaz,G., Stancampiano,R., Diana,A., Carta,M., Curreli,R., Sarais,L., and Fadda,F. (2002). Vitamin A deficiency produces spatial learning and memory impairment in rats. Neuroscience *115*, 475-482.

Cohan,C.S., Connor,J.A., and Kater,S.B. (1987). Electrically and chemically mediated increases in intracellular calcium in neuronal growth cones. J. Neurosci. *7*, 3588-3599.

Connor,J.A. (1986). Digital imaging of free calcium changes and of spatial gradients in growing processes in single, mammalian central nervous system cells. Proc. Natl. Acad. Sci. U. S. A *83*, 6179-6183.

Coulon,P., Herr,D., Kanyshkova,T., Meuth,P., Budde,T., and Pape,H.C. (2009). Burst discharges in neurons of the thalamic reticular nucleus are shaped by calcium-induced calcium release. Cell Calcium *46*, 333-346.

Crair,M.C. (1999). Neuronal activity during development: permissive or instructive? Curr. Opin. Neurobiol. *9*, 88-93.

De,K.J., De,K.N., Merkx,H., Vervaeck,M., and Herroelen,L. (1992). Serum concentrations of vitamins A and E and early outcome after ischaemic stroke. Lancet *339*, 1562-1565.

De,T.H., Tiollais,P., and Dejean,A. (1990). The retinoic acid receptors. Nouv. Rev. Fr. Hematol. *32*, 30-32.

Del Rincon,S.V. and Scadding,S.R. (2002). Retinoid antagonists inhibit normal patterning during limb regeneration in the axolotl, Ambystoma mexicanum. J. Exp. Zool. *292*, 435-443.

Dmetrichuk,J.M., Carlone,R.L., Jones,T.R., Vesprini,N.D., and Spencer,G.E. (2008). Detection of endogenous retinoids in the molluscan CNS and characterization of the trophic and tropic actions of 9-cis retinoic acid on isolated neurons. J. Neurosci. *28*, 13014-13024.

Dmetrichuk,J.M., Carlone,R.L., and Spencer,G.E. (2006). Retinoic acid induces neurite outgrowth and growth cone turning in invertebrate neurons. Dev. Biol. *294*, 39-49.

Dmetrichuk,J.M., Spencer,G.E., and Carlone,R.L. (2005). Retinoic acid-dependent attraction of adult spinal cord axons towards regenerating newt limb blastemas in vitro. Dev. Biol. *281*, 112-120.

Dolle,P. (2009). Developmental expression of retinoic acid receptors (RARs). Nucl. Recept. Signal. *7*, e006.

Duprez,E., Lillehaug,J.R., Gaub,M.P., and Lanotte,M. (1996). Differential changes of retinoid-X-receptor (RXR alpha) and its RAR alpha and PML-RAR alpha partners induced by retinoic acid and cAMP distinguish maturation sensitive and resistant t(15;17) promyelocytic leukemia NB4 cells. Oncogene *12*, 2443-2450.

Ebisawa,M., Umemiya,H., Ohta,K., Fukasawa,H., Kawachi,E., Christoffel,G., Gronemeyer,H., Tsuji,M., Hashimoto,Y., Shudo,K., and Kagechika,H. (1999). Retinoid X receptor-antagonistic diazepinylbenzoic acids. Chem. Pharm. Bull. (Tokyo) *47*, 1778-1786.

Elizalde,C., Campa,V.M., Caro,M., Schlangen,K., Aransay,A.M., Vivanco,M., and Kypta,R.M. (2011). Distinct roles for Wnt-4 and Wnt-11 during retinoic acid-induced neuronal differentiation. Stem Cells *29*, 141-153.

Farrar,N.R., Dmetrichuk,J.M., Carlone,R.L., and Spencer,G.E. (2009). A novel, nongenomic mechanism underlies retinoic acid-induced growth cone turning. J. Neurosci. *29*, 14136-14142.

Feisst,C., Albert,D., Steinhilber,D., and Werz,O. (2005). The aminosteroid phospholipase C antagonist U-73122 (1-[6-[[17-beta-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrro le-2,5-dione) potently inhibits human 5-lipoxygenase in vivo and in vitro. Mol. Pharmacol. *67*, 1751-1757.

Feng,Z.P., Zhang,Z., van Kesteren,R.E., Straub,V.A., van,N.P., Jin,K., Nejatbakhsh,N., Goldberg,J.I., Spencer,G.E., Yeoman,M.S., Wildering,W., Coorssen,J.R., Croll,R.P., Buck,L.T., Syed,N.I., and Smit,A.B. (2009). Transcriptome analysis of the central nervous system of the mollusc Lymnaea stagnalis. BMC. Genomics *10*, 451.

Fields,R.D., Lee,P.R., and Cohen,J.E. (2005). Temporal integration of intracellular Ca2+ signaling networks in regulating gene expression by action potentials. Cell Calcium *37*, 433-442.

Gao,Z.Y., Xu,G., Stwora-Wojczyk,M.M., Matschinsky,F.M., Lee,V.M., and Wolf,B.A. (1998). Retinoic acid induction of calcium channel expression in human NT2N neurons. Biochem. Biophys. Res. Commun. *247*, 407-413.

Geny,B., Cost,H., Barreau,P., Basset,M., Le,P.C., Abita,J.P., and Cockcroft,S. (1991). The differentiating agent, retinoic acid, causes an early inhibition of inositol lipid-specific phospholipase C activity in HL-60 cells. Cell Signal. *3*, 11-23.

Gjertsen,B.T., Mellgren,G., Otten,A., Maronde,E., Genieser,H.G., Jastorff,B., Vintermyr,O.K., McKnight,G.S., and Doskeland,S.O. (1995). Novel (Rp)-cAMPS analogs as tools for inhibition of cAMP-kinase in cell culture. Basal cAMP-kinase activity modulates interleukin-1 beta action. J. Biol. Chem. *270*, 20599-20607.

Gupta,P., Ho,P.C., Huq,M.M., Ha,S.G., Park,S.W., Khan,A.A., Tsai,N.P., and Wei,L.N. (2008). Retinoic acid-stimulated sequential phosphorylation, PML recruitment, and SUMOylation of nuclear receptor TR2 to suppress Oct4 expression. Proc. Natl. Acad. Sci. U. S. A *105*, 11424-11429.

Haase,I., Liesegang,C., Henz,B.M., and Rosenbach,T. (1997). Retinoic acid attenuates phospholipase C-mediated signaling in HaCaT keratinocytes. Arch. Dermatol. Res. *289*, 533-539.

Hamakawa,T., Woodin,M.A., Bjorgum,M.C., Painter,S.D., Takasaki,M., Lukowiak,K., Nagle,G.T., and Syed,N.I. (1999). Excitatory synaptogenesis between identified Lymnaea neurons requires extrinsic trophic factors and is mediated by receptor tyrosine kinases. J. Neurosci. *19*, 9306-9312.

Han,G., Chang,B., Connor,M.J., and Sidell,N. (1995). Enhanced potency of 9-cis versus all-trans-retinoic acid to induce the differentiation of human neuroblastoma cells. Differentiation *59*, 61-69.

Han,Y.H., Zhou,H., Kim,J.H., Yan,T.D., Lee,K.H., Wu,H., Lin,F., Lu,N., Liu,J., Zeng,J.Z., and Zhang,X.K. (2009). A unique cytoplasmic localization of retinoic acid receptor-gamma and its regulations. J. Biol. Chem. *284*, 18503-18514.

Harris-Warrick,R.M. and Johnson,B.R. (1987). Potassium channel blockade induces rhythmic activity in a conditional burster neuron. Brain Res. *416*, 381-386.

Herring,T.L., Slotin,I.M., Baltz,J.M., and Morris,C.E. (1998). Neuronal swelling and surface area regulation: elevated intracellular calcium is not a requirement. Am. J. Physiol *274*, C272-C281.

Hiroshi Nakamura,S.K.S.K.A.U.a.E.I. and Bregestovski,P.D. (1977). PKA-Dependent Regulation of Synaptic Enhancement between a Buccal Motor Neuron and Its Regulatory Interneuron in Lymnaea stagnalis. Acta Physiol Acad. Sci. Hung. *49*, 221-230.

Honda,M., Hamazaki,T.S., Komazaki,S., Kagechika,H., Shudo,K., and Asashima,M. (2005). RXR agonist enhances the differentiation of cardiomyocytes derived from embryonic stem cells in serum-free conditions. Biochem. Biophys. Res. Commun. *333*, 1334-1340.

Hull,P.R. and D'Arcy,C. (2003). Isotretinoin use and subsequent depression and suicide: presenting the evidence. Am. J. Clin. Dermatol. *4*, 493-505.

Hyatt,G.A., Schmitt,E.A., Marsh-Armstrong,N.R., and Dowling,J.E. (1992). Retinoic acid-induced duplication of the zebrafish retina. Proc. Natl. Acad. Sci. U. S. A *89*, 8293-8297.

Ibarretxe,G., Perrais,D., Jaskolski,F., Vimeney,A., and Mulle,C. (2007a). Fast regulation of axonal growth cone motility by electrical activity. J. Neurosci. *27*, 7684-7695.

Ibarretxe,G., Perrais,D., Jaskolski,F., Vimeney,A., and Mulle,C. (2007b). Fast regulation of axonal growth cone motility by electrical activity. J. Neurosci. *27*, 7684-7695.

Jetten,A.M. and Shirley,J.E. (1985). Retinoids antagonize the induction of ornithine decarboxylase activity by phorbol esters and phospholipase C in rat tracheal epithelial cells. J. Cell Physiol *123*, 386-394.

Jong,L., Lehmann,J.M., Hobbs,P.D., Harlev,E., Huffman,J.C., Pfahl,M., and Dawson,M.I. (1993). Conformational effects on retinoid receptor selectivity. 1. Effect of 9-double bond geometry on retinoid X receptor activity. J. Med. Chem. *36*, 2605-2613.

Kagechika,H. (2002). Novel synthetic retinoids and separation of the pleiotropic retinoidal activities. Curr. Med. Chem. *9*, 591-608.

Kamei,Y., Kawada,T., Kazuki,R., and Sugimoto,E. (1993). Retinoic acid receptor gamma 2 gene expression is up-regulated by retinoic acid in 3T3-L1 preadipocytes. Biochem. J. *293 (Pt 3)*, 807-812.

Kampmann,E. and Mey,J. (2007). Retinoic acid enhances Erk phosphorylation in the chick retina. Neurosci. Lett. *426*, 18-22.

Katsuki,H., Kurimoto,E., Takemori,S., Kurauchi,Y., Hisatsune,A., Isohama,Y., Izumi,Y., Kume,T., Shudo,K., and Akaike,A. (2009). Retinoic acid receptor stimulation protects midbrain dopaminergic neurons from inflammatory degeneration via BDNF-mediated signaling. J. Neurochem. *110*, 707-718.

Katz,L.C. and Shatz,C.J. (1996). Synaptic activity and the construction of cortical circuits. Science *274*, 1133-1138.

Holodenko,R., Kholodenko,I., Sorokin,V., Tolmazova,A., Sazonova,O., and Buzdin,A. (2007). Anti-apoptotic effect of retinoic acid on retinal progenitor cells mediated by a protein kinase A-dependent mechanism. Cell Res. *17*, 151-162.

Kim,S.W., Hong,J.S., Ryu,S.H., Chung,W.C., Yoon,J.H., and Koo,J.S. (2007). Regulation of mucin gene expression by CREB via a nonclassical retinoic acid signaling pathway. Mol. Cell Biol. *27*, 6933-6947.

Knoferle,J., Koch,J.C., Ostendorf,T., Michel,U., Planchamp,V., Vutova,P., Tonges,L., Stadelmann,C., Bruck,W., Bahr,M., and Lingor,P. (2010). Mechanisms of acute axonal degeneration in the optic nerve in vivo. Proc. Natl. Acad. Sci. U. S. A *107*, 6064-6069.

Koch,J.C., Knoferle,J., Tonges,L., Ostendorf,T., Bahr,M., and Lingor,P. (2010). Acute axonal degeneration in vivo is attenuated by inhibition of autophagy in a calcium-dependent manner. Autophagy. *6*.

Koert,C.E., Spencer,G.E., van,M.J., Li,K.W., Geraerts,W.P., Syed,N.I., Smit,A.B., and van Kesteren,R.E. (2001). Functional implications of neurotransmitter expression during axonal regeneration: serotonin, but not peptides, auto-regulate axon growth of an identified central neuron. J. Neurosci. *21*, 5597-5606.

Konta,T., Xu,Q., Furusu,A., Nakayama,K., and Kitamura,M. (2001). Selective roles of retinoic acid receptor and retinoid x receptor in the suppression of apoptosis by all-trans-retinoic acid. J. Biol. Chem. *276*, 12697-12701.

Kot-Leibovich,H. and Fainsod,A. (2009). Ethanol induces embryonic malformations by competing for retinaldehyde dehydrogenase activity during vertebrate gastrulation. Dis. Model. Mech. *2*, 295-305.

Krezel,W., Ghyselinck,N., Samad,T.A., Dupe,V., Kastner,P., Borrelli,E., and Chambon,P. (1998). Impaired locomotion and dopamine signaling in retinoid receptor mutant mice. Science *279*, 863-867.

Kumar,A., Singh,C.K., DiPette,D.D., and Singh,U.S. (2010). Ethanol impairs activation of retinoic acid receptors in cerebellar granule cells in a rodent model of fetal alcohol spectrum disorders. Alcohol Clin. Exp. Res. *34*, 928-937.

Lacchini,A.H., Davies,A.J., Mackintosh,D., and Walker,A.J. (2006). Beta-1, 3-glucan modulates PKC signalling in Lymnaea stagnalis defence cells: a role for PKC in H2O2 production and downstream ERK activation. J. Exp. Biol. *209*, 4829-4840.

Laserna,E.J., Valero,M.L., Sanz,L., del Pino,M.M., Calvete,J.J., and Barettino,D. (2009). Proteomic analysis of phosphorylated nuclear proteins underscores novel roles for rapid actions of retinoic acid in the regulation of mRNA splicing and translation. Mol. Endocrinol. *23*, 1799-1814.

Launay,S., Gianni,M., Diomede,L., Machesky,L.M., Enouf,J., and Papp,B. (2003). Enhancement of ATRA-induced cell differentiation by inhibition of calcium accumulation into the endoplasmic reticulum: cross-talk between RAR alpha and calcium-dependent signaling. Blood *101*, 3220-3228.

Lautermilch,N.J. and Spitzer,N.C. (2000). Regulation of calcineurin by growth cone calcium waves controls neurite extension. J. Neurosci. *20*, 315-325.

Lawson,N.D., Zain,M., Zibello,T., Picciotto,M.R., Nairn,A.C., and Berliner,N. (1999). Modulation of a calcium/calmodulin-dependent protein kinase cascade by retinoic acid during neutrophil maturation. Exp. Hematol. *27*, 1682-1690.

Lee,G.S., Kochhar,D.M., and Collins,M.D. (2004). Retinoid-induced limb malformations. Curr. Pharm. Des *10*, 2657-2699.

Lee,T.K. and Syed,N.I. (2004). Transplantation and restoration of functional synapses between an identified neuron and its targets in the intact brain of Lymnaea stagnalis. Synapse *51*, 186-193.

Liao,Y.P., Ho,S.Y., and Liou,J.C. (2004). Non-genomic regulation of transmitter release by retinoic acid at developing motoneurons in Xenopus cell culture. J. Cell Sci. *117*, 2917-2924.

Liou,J.C., Ho,S.Y., Shen,M.R., Liao,Y.P., Chiu,W.T., and Kang,K.H. (2005). A rapid, nongenomic pathway facilitates the synaptic transmission induced by retinoic acid at the developing synapse. J. Cell Sci. *118*, 4721-4730.

Lukowiak,K., Haque,Z., Spencer,G., Varshay,N., Sangha,S., and Syed,N. (2003). Long-term memory survives nerve injury and the subsequent regeneration process. Learn. Mem. *10*, 44-54.

Maden,M. (2007). Retinoic acid in the development, regeneration and maintenance of the nervous system. Nat. Rev. Neurosci. *8*, 755-765.

Maden,M. and Hind,M. (2003). Retinoic acid, a regeneration-inducing molecule. Dev. Dyn. *226*, 237-244.

Maghsoodi,B., Poon,M.M., Nam,C.I., Aoto,J., Ting,P., and Chen,L. (2008). Retinoic acid regulates RARalpha-mediated control of translation in dendritic RNA granules during homeostatic synaptic plasticity. Proc. Natl. Acad. Sci. U. S. A *105*, 16015-16020.

Mao,C.T., Li,T.Y., Qu,P., Zhao,Y., Wang,R., and Liu,Y.X. (2006). [Effects of early intervention on learning and memory in young rats of marginal vitamin A deficiency and it's mechanism]. Zhonghua Er. Ke. Za Zhi. *44*, 15-20.

Mattson,M.P., Dou,P., and Kater,S.B. (1988a). Outgrowth-regulating actions of glutamate in isolated hippocampal pyramidal neurons. J. Neurosci. *8*, 2087-2100.

Mattson,M.P. and Kater,S.B. (1987). Calcium regulation of neurite elongation and growth cone motility. J. Neurosci. *7*, 4034-4043.

Mattson,M.P., Taylor-Hunter,A., and Kater,S.B. (1988b). Neurite outgrowth in individual neurons of a neuronal population is differentially regulated by calcium and cyclic AMP. J. Neurosci. *8*, 1704-1711.

McClellan,A.D., Kovalenko,M.O., Benes,J.A., and Schulz,D.J. (2008). Spinal cord injury induces changes in electrophysiological properties and ion channel expression of reticulospinal neurons in larval lamprey. J. Neurosci. *28*, 650-659.

McCobb,D.P., Cohan,C.S., Connor,J.A., and Kater,S.B. (1988). Interactive effects of serotonin and acetylcholine on neurite elongation. Neuron *1*, 377-385.

McConkey,G.A., Rogers,M.J., and McCutchan,T.F. (1997). Inhibition of Plasmodium falciparum protein synthesis. Targeting the plastid-like organelle with thiostrepton. J. Biol. Chem. *272*, 2046-2049.

McGrane,M.M. (2007). Vitamin A regulation of gene expression: molecular mechanism of a prototype gene. J. Nutr. Biochem. *18*, 497-508.

Meeks,R.G., Zaharevitz,D., and Chen,R.F. (1981). Membrane effects of retinoids: possible correlation with toxicity. Arch. Biochem. Biophys. *207*, 141-147.

Mey,J. and McCaffery,P. (2004). Retinoic acid signaling in the nervous system of adult vertebrates. Neuroscientist. *10*, 409-421.

Ming,G., Henley,J., Tessier-Lavigne,M., Song,H., and Poo,M. (2001). Electrical activity modulates growth cone guidance by diffusible factors. Neuron *29*, 441-452.

Molnar,G., Salanki,J., and Kiss,T. (2004). Cadmium inhibits GABA-activated ion currents by increasing intracellular calcium level in snail neurons. Brain Res. *1008*, 205-211.

Nagashima,M., Sakurai,H., Mawatari,K., Koriyama,Y., Matsukawa,T., and Kato,S. (2009). Involvement of retinoic acid signaling in goldfish optic nerve regeneration. Neurochem. Int. *54*, 229-236.

Ochoa,W.F., Torrecillas,A., Fita,I., Verdaguer,N., Corbalan-Garcia,S., and Gomez-Fernandez,J.C. (2003). Retinoic acid binds to the C2-domain of protein kinase C(alpha). Biochemistry *42*, 8774-8779.

Oh,Y.I., Kim,J.H., and Kang,C.W. (2010). Oxidative stress in MCF-7 cells is involved in the effects of retinoic acid-induced activation of protein kinase C-delta on insulin-like growth factor-I secretion and synthesis. Growth Horm. IGF. Res. *20*, 101-109.

Patatanian,E. and Thompson,D.F. (2008). Retinoic acid syndrome: a review. J. Clin. Pharm. Ther. *33*, 331-338.

Poon,M.M. and Chen,L. (2008). Retinoic acid-gated sequence-specific translational control by RARalpha. Proc. Natl. Acad. Sci. U. S. A *105*, 20303-20308.

Quenech'Du,N., Ruchaud,S., Khelef,N., Guiso,N., and Lanotte,M. (1998). A sustained increase in the endogenous level of cAMP reduces the retinoid concentration required for APL cell maturation to near physiological levels. Leukemia *12*, 1829-1833.

Rand, C., Carter, R.Carlone, and G.Spencer. (2011). The role of retinoid receptors in growth cone turning of invertebrate and vertebrate neurons. 862.07. Society for Neuroscience Abstract. Washington, DC.

Reijntjes,S., Francis-West,P., and Mankoo,B.S. (2010). Retinoic acid is both necessary for and inhibits myogenic commitment and differentiation in the chick limb. Int. J. Dev. Biol. *54*, 125-134.

Reimer,M.M., Kuscha,V., Wyatt,C., Sorensen,I., Frank,R.E., Knuwer,M., Becker,T., and Becker,C.G. (2009). Sonic hedgehog is a polarized signal for motor neuron regeneration in adult zebrafish. J. Neurosci. *29*, 15073-15082.

Reynolds,I.J., Wagner,J.A., Snyder,S.H., Thayer,S.A., Olivera,B.M., and Miller,R.J. (1986). Brain voltage-sensitive calcium channel subtypes differentiated by omega-conotoxin fraction GVIA. Proc. Natl. Acad. Sci. U. S. A *83*, 8804-8807.

Ribot,J., Felipe,F., Bonet,M.L., and Palou,A. (2004). Retinoic acid administration and vitamin A status modulate retinoid X receptor alpha and retinoic acid receptor alpha levels in mouse brown adipose tissue. Mol. Cell Biochem. *266*, 25-30.

Rochette-Egly,C., Oulad-Abdelghani,M., Staub,A., Pfister,V., Scheuer,I., Chambon,P., and Gaub,M.P. (1995). Phosphorylation of the retinoic acid receptor-alpha by protein kinase A. Mol. Endocrinol. *9*, 860-871.

Romero-Sandoval,E.A., Molina,C., Alique,M., Moreno-Manzano,V., Lucio,F.J., and Herrero,J.F. (2006). Vitamin A active metabolite, all-trans retinoic acid, induces spinal cord sensitization. I. Effects after oral administration. Br. J. Pharmacol. *149*, 56-64.

Sah,P. and Faber,E.S. (2002). Channels underlying neuronal calcium-activated potassium currents. Prog. Neurobiol. *66*, 345-353.

Saito,Y., Okamura,M., Nakajima,S., Hayakawa,K., Huang,T., Yao,J., and Kitamura,M. (2010). Suppression of nephrin expression by TNF-alpha via interfering with the cAMP-retinoic acid receptor pathway. Am. J. Physiol Renal Physiol *298*, F1436-F1444.

Sanchez-Alonso,J.L., Munoz-Cuevas,J., Vicente-Torres,M.A., and Colino,A. (2010). Role of low-voltage-activated calcium current on the firing pattern alterations induced by hypothyroidism in the rat hippocampus. Neuroscience *171*, 993-1005.

Santos,N.C. and Kim,K.H. (2010). Activity of retinoic acid receptor-alpha is directly regulated at its protein kinase A sites in response to follicle-stimulating hormone signaling. Endocrinology *151*, 2361-2372.

Scadding,S.R. and Maden,M. (1994). Retinoic acid gradients during limb regeneration. Dev. Biol. *162*, 608-617.

Scheibenstock,A., Krygier,D., Haque,Z., Syed,N., and Lukowiak,K. (2002). The Soma of RPeD1 must be present for long-term memory formation of associative learning in Lymnaea. J. Neurophysiol. *88*, 1584-1591.

Schrage,K., Koopmans,G., Joosten,E.A., and Mey,J. (2006). Macrophages and neurons are targets of retinoic acid signaling after spinal cord contusion injury. Eur. J. Neurosci. *23*, 285-295.

Shepard,P.D. and Bunney,B.S. (1991). Repetitive firing properties of putative dopamine-containing neurons in vitro: regulation by an apamin-sensitive Ca(2+)-activated K+ conductance. Exp. Brain Res. *86*, 141-150.

Short,A.D., Brown,B.L., and Dobson,P.R. (1991). The effect of retinoic acid on parathyroid hormone- and parathyroid hormone-related peptide-induced intracellular calcium in a rat osteosarcoma cell line, UMR106. J. Endocrinol. *129*, 75-81.

Shyu,R.Y., Huang,S.L., and Jiang,S.Y. (2003). Retinoic acid increases expression of the calcium-binding protein S100P in human gastric cancer cells. J. Biomed. Sci. *10*, 313-319.

Sidell,N., Feng,Y., Hao,L., Wu,J., Yu,J., Kane,M.A., Napoli,J.L., and Taylor,R.N. (2010). Retinoic acid is a cofactor for translational regulation of vascular endothelial growth factor in human endometrial stromal cells. Mol. Endocrinol. *24*, 148-160.

So,P.L., Yip,P.K., Bunting,S., Wong,L.F., Mazarakis,N.D., Hall,S., McMahon,S., Maden,M., and Corcoran,J.P. (2006). Interactions between retinoic acid, nerve growth factor and sonic hedgehog signalling pathways in neurite outgrowth. Dev. Biol. *298*, 167-175.

Suzuki,K., Takahashi,K., Nishimaki-Mogami,T., Kagechika,H., Yamamoto,M., and Itabe,H. (2009). Docosahexaenoic acid induces adipose differentiation-related protein through activation of retinoid x receptor in human choriocarcinoma BeWo cells. Biol. Pharm. Bull. *32*, 1177-1182.

Syed,N.I., Ridgway,R.L., Lukowiak,K., and Bulloch,A.G. (1992). Transplantation and functional integration of an identified respiratory interneuron in Lymnaea stagnalis. Neuron *8*, 767-774.

Syed,N.I. and Winlow,W. (1991). Coordination of locomotor and cardiorespiratory networks of Lymnaea stagnalis by a pair of identified interneurones. J. Exp. Biol. *158*, 37-62.

Takahashi,B., Ohta,K., Kawachi,E., Fukasawa,H., Hashimoto,Y., and Kagechika,H. (2002). Novel retinoid X receptor antagonists: specific inhibition of retinoid synergism in RXR-RAR heterodimer actions. J. Med. Chem. *45*, 3327-3330.

Takamatsu,K., Takano,A., Yakushiji,N., Morishita,K., Matsuura,N., Makishima,M., Ali,H.I., Akaho,E., Tai,A., Sasaki,K., and Kakuta,H. (2008). Reduction of lipophilicity at the lipophilic domain of RXR agonists enables production of subtype preference: RXRalpha-preferential agonist possessing a sulfonamide moiety. ChemMedChem. *3*, 454-460.

Tanaka,M., Tamura,K., and Ide,H. (1996). Citral, an inhibitor of retinoic acid synthesis, modifies chick limb development. Dev. Biol. *175*, 239-247.

Thaller,C., Hofmann,C., and Eichele,G. (1993). 9-cis-retinoic acid, a potent inducer of digit pattern duplications in the chick wing bud. Development *118*, 957-965.

Thatcher,J.E. and Isoherranen,N. (2009). The role of CYP26 enzymes in retinoic acid clearance. Expert. Opin. Drug Metab Toxicol. *5*, 875-886.

Theodosiou,M., Laudet,V., and Schubert,M. (2010). From carrot to clinic: an overview of the retinoic acid signaling pathway. Cell Mol. Life Sci. *67*, 1423-1445.

Tiwari,M., Chandra,R., Das,S.K., and Prakash,S. (2007). Tin mesoporphyrin in conjunction with retinoic acid reverses the retinoic acid induced enhancement of phospholipase A(2) activity in vivo in rats. Artif. Cells Blood Substit. Immobil. Biotechnol. *35*, 275-285.

Tonini,R., Mancinelli,E., Balestrini,M., Mazzanti,M., Martegani,E., Ferroni,A., Sturani,E., and Zippel,R. (1999). Expression of Ras-GRF in the SK-N-BE neuroblastoma accelerates retinoic-acid-induced neuronal differentiation and increases the functional expression of the IRK1 potassium channel. Eur. J. Neurosci. *11*, 959-966.

Ulusoy,G.K., Celik,T., Kayir,H., Gursoy,M., Isik,A.T., and Uzbay,T.I. (2011). Effects of pioglitazone and retinoic acid in a rotenone model of Parkinson's disease. Brain Res. Bull.

Umemiya,H., Fukasawa,H., Ebisawa,M., Eyrolles,L., Kawachi,E., Eisenmann,G., Gronemeyer,H., Hashimoto,Y., Shudo,K., and Kagechika,H. (1997). Regulation of retinoidal actions by diazepinylbenzoic acids. Retinoid synergists which activate the RXR-RAR heterodimers. J. Med. Chem. *40*, 4222-4234.

Vandersea,M.W., Fleming,P., McCarthy,R.A., and Smith,D.G. (1998). Fin duplications and deletions induced by disruption of retinoic acid signaling. Dev. Genes Evol. *208*, 61-68.

Vatanparast,J. and Janahmadi,M. (2009). Contribution of apamin-sensitive SK channels to the firing precision but not to the slow afterhyperpolarization and spike frequency adaptation in snail neurons. Brain Res. *1255*, 57-66.

White,R.J., Nie,Q., Lander,A.D., and Schilling,T.F. (2007). Complex regulation of cyp26a1 creates a robust retinoic acid gradient in the zebrafish embryo. PLoS. Biol. *5*, e304.

Wiegert,J.S. and Bading,H. (2010). Activity-dependent calcium signaling and ERK-MAP kinases in neurons: A link to structural plasticity of the nucleus and gene transcription regulation. Cell Calcium.

Wietrzych,M., Meziane,H., Sutter,A., Ghyselinck,N., Chapman,P.F., Chambon,P., and Krezel,W. (2005). Working memory deficits in retinoid X receptor gamma-deficient mice. Learn. Mem. *12*, 318-326.

Wong,R.G., Hadley,R.D., Kater,S.B., and Hauser,G.C. (1981). Neurite outgrowth in molluscan organ and cell cultures: the role of conditioning factor(s). J. Neurosci. *1*, 1008-1021.

Wu,Y., Mou,Z., Li,J., Zhou,W., Wei,B., and Zou,L. (2004). Identification of a S100 calcium-binding protein expressed in HL-60 cells treated with all-trans retinoic acid by two-dimensional electrophoresis and mass spectrometry. Leuk. Res. *28*, 203-207.

Xiao,Y.F., Wright,S.N., Wang,G.K., Morgan,J.P., and Leaf,A. (1998). Fatty acids suppress voltage-gated Na+ currents in HEK293t cells transfected with the alpha-subunit of the human cardiac Na+ channel. Proc. Natl. Acad. Sci. U. S. A *95*, 2680-2685.

Yamuy,J., Pose,I., Pedroarena,C., Morales,F.R., and Chase,M.H. (2000). Neurotrophin-induced rapid enhancement of membrane potential oscillations in mesencephalic trigeminal neurons. Neuroscience *95*, 1089-1100.

Zanotto-Filho,A., Cammarota,M., Gelain,D.P., Oliveira,R.B., Delgado-Canedo,A., Dalmolin,R.J., Pasquali,M.A., and Moreira,J.C. (2008). Retinoic acid induces apoptosis by a non-classical mechanism of ERK1/2 activation. Toxicol. In Vitro *22*, 1205-1212.

Zeng,J.Z., Sun,D.F., Wang,L., Cao,X., Qi,J.B., Yang,T., Hu,C.Q., Liu,W., and Zhang,X.K. (2006). Hypericum sampsonii induces apoptosis and nuclear export of retinoid X receptor-alpha. Carcinogenesis *27*, 1991-2000.

Zhang,D.Q. and McMahon,D.G. (2000). Direct gating by retinoic acid of retinal electrical synapses. Proc. Natl. Acad. Sci. U. S. A *97*, 14754-14759.

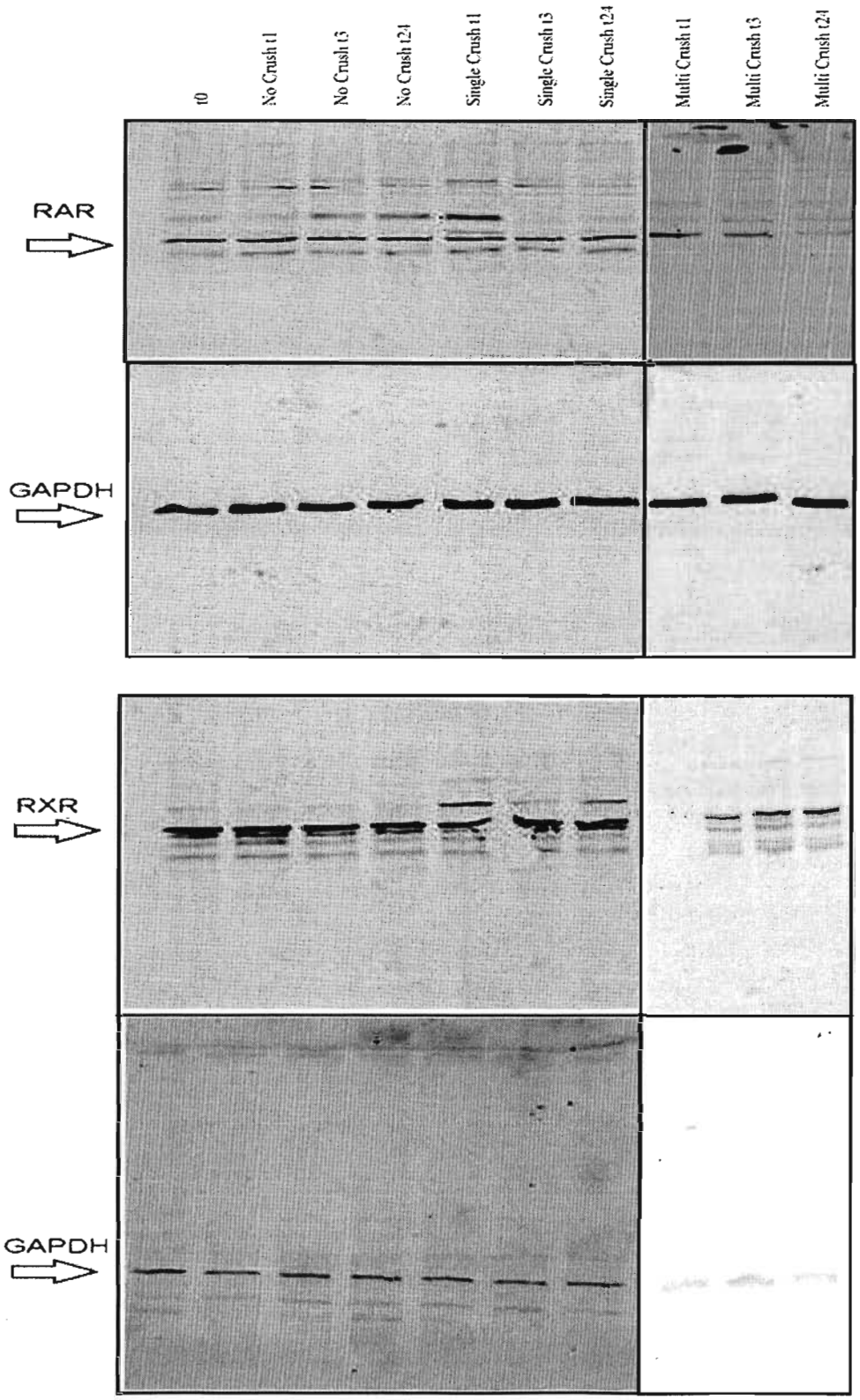
Zhelyaznik,N. and Mey,J. (2006). Regulation of retinoic acid receptors alpha, beta and retinoid X receptor alpha after sciatic nerve injury. Neuroscience *141*, 1761-1774.

Zhelyaznik,N., Schrage,K., McCaffery,P., and Mey,J. (2003). Activation of retinoic acid signalling after sciatic nerve injury: up-regulation of cellular retinoid binding proteins. Eur. J. Neurosci. *18*, 1033-1040.

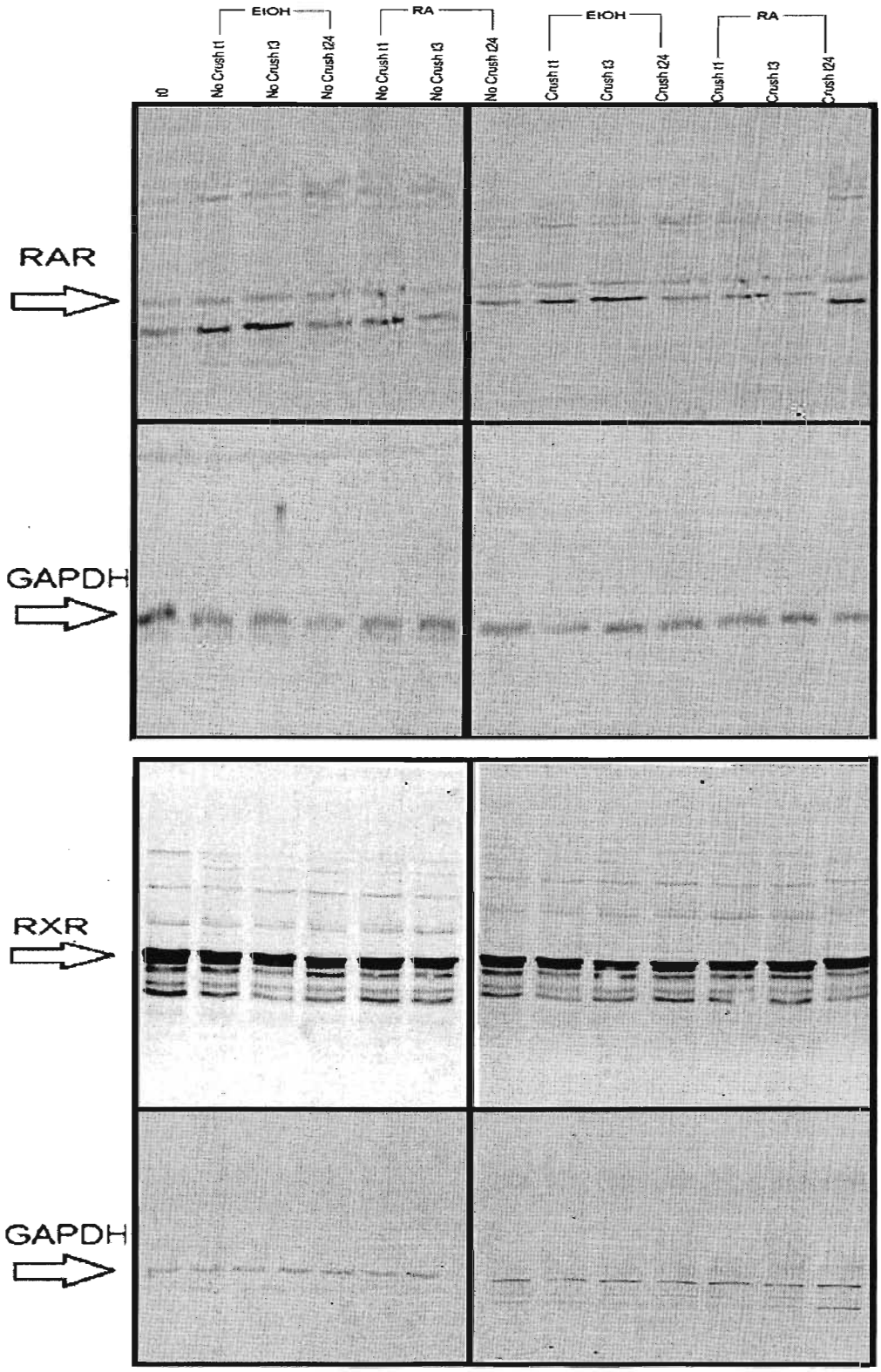
Zile,M.H. (2001). Function of vitamin A in vertebrate embryonic development. J. Nutr. *131*, 705-708.

6.0 Appendix

1.01 - A: Full Western blot of CNS preparations exposed to a single, multiple or no nerve-crush injury for 1 (t1) 3 (t3) or 24 (t24) hours. Arrows indicate the protein band at the expected weight of the RAR, RXR or GAPDH, respectively. Separate blots are boxed for clarity.



1.01– B: Full Western blot of CNS preparations exposed to EtOH or RA in the presence or absence of a nerve-crush injury for 1 (t1) 3 (t3) or 24 (t24) hours. Arrows indicate the protein band at the expected weight of the RAR, RXR or GAPDH, respectively. Separate blots are boxed for clarity.



1.02 – Spike waveform parameters at the 35 and 50 minute time point after exposure to RA or EtOH (control) in VF and RPeD1 neurons from CNS preparations in the presence or absence of a nerve crush for 1 hour. Data are expressed as a percent of baseline, set at 100%. In all cases, no significant differences were found in RA-exposed neurons compared to EtOH-exposed neurons in the same crush condition. (Mann-Whitney t-tests performed).

Crushed CNS Preparations:

35 minutes of exposure	**RPeD1 - EtOH 10µM**	**RPeD1 - RA 10µM**	**VF - EtOH 10µM**	**VF - RA 10µM**
Peak to peak amplitude (%)	95.5 ± 6.7	95.5 ± 3.8	103.8 ± 4.6	93.2 ± 6.5
Rise time (%)	167.9 ± 111.6	86.3 ± 45.3	40.9 ± 19.7	43.2 ± 47.9
Half-amplitude duration (%)	135.2 ± 14.3	127.3 ±7.8	103.4 ± 6.2	95.5 ±6.4
Decay time (%)	143.7 ± 15.4	143.0 ± 9.3	107.0 ± 7.2	108.8 ± 9.9
50 minutes of exposure				
Peak to peak amplitude (%)	99.8 ± 6.8	94.9 ± 7.7	102.7 ± 7.6	91.4 ± 5.4
Rise time (%)	157.6 ± 49.9	359.7 ± 182.0	345.4 ± 115.7	409.4 ± 253.1
Half-amplitude duration (%)	130.2 ± 12.4	140.0 ± 11.8	103.1 ± 7.6	115.8 ± 12.7
Decay time (%)	133.4 ± 13.4	150.9 ± 15.2	103.3 ± 8.4	130.9 ± 16.3

Uncrushed CNS Preparations:

35 minutes of exposure	RPeD1 - EtOH 10µM	RPeD1 - RA 10µM	VF - EtOH 10µM	VF - RA 10µM
Peak to peak amplitude (%)	100.5 ± 5.4	96.7 ± 8.1	90.3 ± 4.4	93.3 ± 6.4
Rise time (%)	157.8 ± 40.3	108.1 ± 16.2	234.9 ± 123.7	140.5 ± 53.6
Half-amplitude duration (%)	129.7 ± 7.7	148.1 ± 9.0	108.1 ± 8.4	125.2 ± 15.1
Decay time (%)	126.0 ± 9.4	111.2 ± 19.0	98.4 ± 8.3	113.3 ± 14.4
50 minutes of exposure				
Peak to peak amplitude (%)	101.8 ± 5.7	94.9 ± 7.0	95.4 ± 3.8	99.2 ± 9.8
Rise time (%)	174.6 ± 39.0	189.7 ± 61.6	135.8 ± 41.7	183.7 ± 72.5
Half-amplitude duration (%)	133.7 ± 8.5	150.2 ± 15.3	105.6 ± 10.7	115.1 ± 13.8
Decay time (%)	129.5 ± 8.3	128.5 ± 15.0	115.9 ± 15.6	138.2 ± 19.8

1.03 – Resting membrane potential from VF and RPeD1 neurons taken 10 minutes prior to (pre-exposure) and 60 minutes after exposure to either RA or EtOH (control) in isolated CNS preparations. Note that both VF and RPeD1 neurons were exposed to either RA or EtOH only. In all cases, no significant differences in RA-exposed neurons were found compared to EtOH controls within the same crush condition (Mann-Whitney t-tests performed).

RMP (mV)	EtOH Pre-exposure	60 min after EtOH	RA Pre-exposure	60 min after RA
Crushed CNS - VF	-64.5 ± 3.2	-66.2 ± 3.4	-65.7 ± 2.6	-68 ± 3.3
Uncrushed CNS - VF	-64.4 ±7.7	-65.2 ± 2.8	-64.6 ± 4.7	-65.8 ± 7.7
Crushed CNS - RPeD1	-74.2 ± 2.5	-72.1 ± 4.5	-71.7 ± 2.1	-69.8 ± 2.6
Uncrushed CNS - RPeD1	-76.4 ± 5.5	-69.5 ± 3.7	-78.6 ± 3.2	-82.5± 6.5

1.04 - Spike waveform parameters at the 35 and 50 minute time point after exposure to RA or EtOH (control) in VF and RPeD1 neurons from CNS preparations in the presence or absence of a nerve crush for 24 hours. Data are expressed as a percent of baseline (set at 100%). Significant differences (if indicated) were found in RA-exposed neurons compared to EtOH-exposed neurons in the same crush condition (t-tests performed; * = $p<0.05$).

Crushed CNS Preparations:

35 minutes of exposure	**RPeD1 - EtOH 10µM**	**RPeD1 - RA 10µM**	**VF - EtOH 10µM**	**VF - RA 10µM**
Peak to peak amplitude (%)	94.6 ± 5.5	102.0 ± 4.3	103.7 ± 5.8	87.2 ± 4.8
Rise time (%)	222.6 ± 77.5	126.6 ± 26.8	127.2 ± 28.7	209.3 ± 67.1
Half-amplitude duration (%)	139.5 ± 15.8	113.8 ± 13.5	93.5 ± 7.7	133.7 ± 33.1*
Decay time (%)	148.8 ±15.0	120.4 ± 17.1	93.5 ± 10.7	280.7 ± 90.7*
50 minutes of exposure				
Peak to peak amplitude (%)	91.6 ± 6.2	96.8 ± 6.7	104.7 ± 6.1	86.9 ± 4.4
Rise time (%)	172.5 ± 30.0	217.3 ± 48.1	140.6 ± 33.2	189.4 ± 50.0
Half-amplitude duration (%)	129.7 ± 11.9	131.7 ± 14.3	98.4 ± 6.8	135.3 ± 24.0*
Decay time (%)	133.2 ± 8.9	147.5 ± 11.6	95.3 ± 17.6	169.2 ± 30.8*

Uncrushed CNS Preparations:

35 minutes of exposure	**RPeD1 - EtOH 10µM**	**RPeD1 - RA 10µM**	**VF - EtOH 10µM**	**VF - RA 10µM**
Peak to peak amplitude (%)	99.3 ± 7.9	93.8 ± 4.3	106.7 ± 7.5	99.7 ± 6.4
Rise time (%)	122.0 ± 15.0	241.1 ± 219.8	108.7 ± 39.2	112.19 ± 19.2
Half-amplitude duration (%)	121.5 ± 6.7	132.4 ± 10.4	108.2 ± 39.2	112.2 ± 18.0
Decay time (%)	137.4 ± 7.5	165.8 ± 15.9	65.1 ± 11.2*	154.5 ± 55.2*
50 minutes of exposure				
Peak to peak amplitude (%)	103.0 ±6.7	99.2 ± 4.6	98.5 ±7.2	99.2 ± 4.5
Rise time (%)	105.3 ± 13.2	205.1 ± 80.3	151.4 ± 55.1	125.6 ± 31.6
Half-amplitude duration (%)	115.3 ± 8.0	123.0 ± 10.5	71.0 ± 9.9*	109.1 ± 10.2*
Decay time (%)	134.2 ± 8.0	149.0 ± 11.0	56.5 ± 10.5*	111.4 ± 20.1*

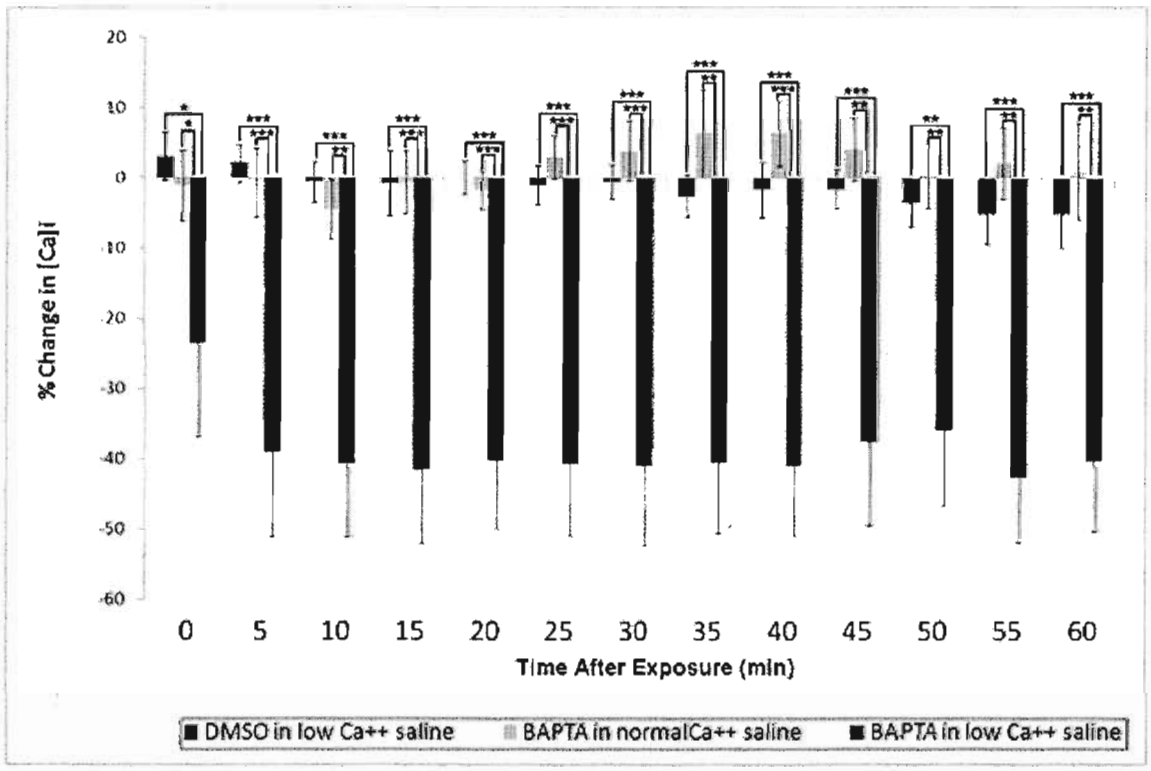
1.05 - Spike waveform parameters at the 15 and 35 minute time point after exposure to RA, RAR agonist TTNPB, RXR agonist Pa024 or vehicle controls (EtOH for RA vehicle, DMSO for agonists vehicle) in VF neurons. Data are expressed as a percent of baseline, set at 100%. Input resistance is expressed as the percent of baseline (set at 100%) 60 minutes after exposure compared to values obtained 10 minutes prior to exposure. RMP expressed as the change (compared to baseline) in raw values 60 minutes after application. In all cases, no significant differences were found. (One way ANOVA performed).

15 minutes of exposure	**EtOH**	**DMSO**	**10μM RA**	**1μM TTNPB**	**10μM Pa024**
Peak to peak amplitude (%)	85.5 ± 4.4	88.7 ± 4.8	88.8 ± 4.2	95.8 ± 3.6	81.1 ± 4.0
Rise time (%)	118.6 ± 48.6	135.8 ± 51.5	54.3 ± 38.2	51.0 ± 28.2	96.7 ± 37.0
35 minutes of exposure					
Peak to peak amplitude (%)	86.1 ± 3.8	83.3 ± 5.4	75.3 ± 3.2	76.7 ± 5.2	85.6 ± 5.2
Rise time (%)	224.7 ± 83.2	184.8 ± 42.7	123.3 ± 30.7	71.6 ± 28.0	134.4 ± 42.6
Input Resistance (%)	135.0 ± 26.5	110.4 ± 10.3	98.1 ± 11.7	101.9 ± 13.7	104.9 ± 6.7
RMP (change in mV)	3.3 ± 2.4	0.7 ± 2.6	6.0 ± 3.6	-9.0 ± 6.6	-1.4 ± 3.2

1.06 - Spike waveform parameters at the 15and 35 minute time point after exposure to RA, RAR antagonist LE540, RXR agoists PA542 and HX531 or vehicle controls (DMSO incubation followed by EtOH exposure) in VF neurons. Data are expressed as a percent of baseline (set at 100%). Input resistance is expressed as the percent change 60 minutes after exposure compared to values obtained 10 minutes prior to exposure (baseline set at 100%). RMP expressed as the change (compared to baseline) in raw values 60 minutes after application. Note that no cells fired beyond 30 minutes for PA024-exposed neurons, and thus no data is shown for this time point. In all cases, no significant differences were found. (One way ANOVA performed).

15 minutes of exposure	**DMSO + EtOH**	**10µM RA**	**1µM LE540 + RA**	**10µM PA542 + RA**	**1µM HX531 + RA**
Peak to peak amplitude (%)	84.7 ± 4.4	88.8 ± 4.2	81.2 ± 2.7	90.0 ± 3.5	87.0 ± 5.0
Rise time (%)	175.9 ± 72.3	54.3 ± 38.2	60.4 ± 23.2	30.3 ± 27.8	9.5 ± 10.5
35 minutes of exposure					
Peak to peak amplitude (%)	83.1 ± 4.9	75.3 ± 3.2	76.8 ± 6.0	---	85.6 ± 8.6
Rise time (%)	123.8 ± 42.8	123.3 ± 30.7	141.3 ± 63.2	---	67.4 ± 21.2
Input Resistance (%)	110.4 ± 8.3	98.1 ± 11.7	135.3 ± 20.1	105.0 ± 6.7	106.8 ± 13.9
RMP (change in mV)	0.7 ± 2.6	6.0 ± 3.6	-9.1 ± 6.6	-2.0 ± 5.0	3.3 ± 2.4

1.07 – BAPTA-AM -induced decreases in intracellular calcium only occur in the absence of extracellular calcium.



Neurons exposed to BAPTA-AM in the presence of normal calcium saline (4.1mM) do not differ from DMSO controls whereas BAPTA-AM -exposed neurons cultured in calcium free saline displayed significantly less intracellular calcium. Two way repeated measures ANOVA performed. (*$p<0.05$, **$p<0.01$, ***$p<0.001$)

1.08 – EC_{50} (in the case of agonists) and IC_{50} (in the case of inhibitors or antagonists) of pharmacological agents used within the studies of this thesis. In all cases, the concentrations of agents used in the studies performed in this thesis are higher than the values reported in other organisms required to elicit half activation (in the case of agonist) or half inactivation (in the case of antagonist or inhibitor) of their intended target.

Inhibitor, Agonist or Antagonist	**Concentration used in thesis**	**EC_{50} (*) or IC_{50} (-)**	**Organism for which EC_{50} or IC_{50} was calculated**	**Source**
Adenosine- 3', 5'-cyclic monophos-phorothioate (PKA inhibitor)	10μM	1uM (-)	rabbit muscle fiber	(Gjertsen et al., 1995)
U 73211 (PLC inhibitor)	20μM	10μM (-)	human platelets	(Feisst et al., 2005)
Anisomycin	45μM	0.5μM (-)	human erythrocytes	(McConkey et al., 1997)
Cadmium	30μM	1μM (-)	rat CNS	(Reynolds et al., 1986)
TTNPB	1μM	85nM (*)	Human promyelocytic leukemia (HL-60) cells	(Jong et al., 1993)
PA024	10μM	3.3nM (*)	Human promyelocytic leukemia (HL-60) cells	(Takamatsu et al., 2008)
LE540	1μM	3.6nM (-)	Human promyelocytic leukemia (HL-60) cells	(Umemiya et al., 1997)
PA452	10μM	100nM (-)	Human promyelocytic leukemia (HL-60) cells	(Takahashi et al., 2002)
HX531	1μM	1.8nM (-)	Human promyelocytic leukemia (HL-60) cells	(Ebisawa et al., 1999)
Apamin	10μM	3.3nM (-)	Monkey kidney (COS) cell line	(Sah and Faber, 2002)
atRA	1 - 10μM	0.2-0.7 nM	Mouse RAR expressed in monkey kidney (COS) cell line	(Allenby et al. 1993)
9*cis* RA	10μM	14.1 - 18.8 nM (*)	Mouse RXR expressed in monkey kidney (COS) cell line	(Allenby et al. 1993)